Transcriptional profiling of epidermal differentiation

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Departments of 1Dermatology and 4Biochemistry, and the 5NYU Cancer Institute, New York University School of Medicine, New York, New York; 2Dermatology Department at the Institute of Clinical Medicine, Tsukuba University, Tsukuba, Ibaraki; and 3Department of Dermatology, Faculty of Medicine, University of Tokyo, Tokyo, Japan

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Radoja, Nada, Alix Gazel, Tomohiro Banno, Shoichiro Yano, and Miroslav Blumenberg. Transcriptional profiling of epidermal differentiation. Physiol Genomics 27: 65–78, 2006. First published July 5, 2006; doi:10.1152/physiolgenomics.00031.2006.—In epidermal differentiation basal keratinocytes detach from the basement membrane, stop proliferating, and express a new set of structural proteins and enzymes, which results in an impermeable protein/lipid barrier that protects us. To define the transcriptional changes essential for this process, we purified large quantities of basal and suprabasal cells from human epidermis, using the expression of β4 integrin as the discriminating factor. The expected expression differences in cytoskeletal, cell cycle, and adhesion genes confirmed the effective separation of the cell populations. Using DNA microarray chips, we comprehensively identify the differences in genes expressed in basal and differentiating layers of the epidermis, including the ECM components produced by the basal cells, the proteases in both the basal and suprabasal cells, and the lipid and steroid metabolism enzymes in suprabasal cells responsible for the permeability barrier. We identified the signaling pathways specific for the two populations and found two previously unknown paracrine and one juxtacrine signaling pathway operating between the basal and suprabasal cells. Furthermore, using specific expression signatures, we identified a new set of late differentiation markers and mapped their chromosomal loci, as well as a new set of melanocyte-specific markers. The data represent a quantum jump in understanding the mechanisms of epidermal differentiation.

basal cells; epidermal differentiation complex; integrins; keratinization; melanocytes; microarrays

DIFFERENTIATION IS THE PROCESS by which progenitor cells acquire functional capabilities. This process, perhaps most studied in lymphoid cells, is ideally suited for global transcriptional profiling because it comprises large transcriptional changes, as cells convert from rapidly proliferating to functionally specialized phenotypes. Indeed, microarrays have been used to study the differentiation processes in human cell lines induced to differentiate along a certain pathway by in vitro treatments with various agents (13, 45). However, so far, no such studies of normally differentiating human cells in vivo have been reported, perhaps because of difficulties in obtaining sufficient quantities of human cell populations at specific stages of differentiation. Epidermis is an ideal target tissue for studies of differentiation because both the progenitor and the differentiating cells can be easily identified and purified in sufficient quantities. Therefore, we decided to develop comprehensive transcriptional profiles of human epidermal basal cells and their differentiated descendants, using large oligonucleotide microarrays.

Four layers can be distinguished morphologically in healthy epidermis, stratum basale, stratum spinosum, stratum granulosum, and stratum corneum (9, 23). The function of stratum basale is to proliferate and to keep the epidermis firmly anchored to the basal lamina. The function of stratum spinosum is to start the differentiation process by producing new cell-cell attachments, cytoskeleton, etc. The function of stratum granulosum is to produce cornified envelope proteins, crosslinking enzymes and appropriate lipids. In stratum corneum, the proteins are crosslinked, lipids are extruded, and the epidermal barrier is formed.

In healthy epidermis, only the basal layer contains mitotic cells. The keratinocytes of the basal layer are characterized by tightly packed palisaded appearance and a position immediately attached to the basal lamina. The cytoskeleton in basal layer contains keratins K5, K14, and small amounts of K15; their function is to anchor the epidermis firmly to its substractum (66). These keratins attach to hemidesmosomes through α6β4 integrins, the laminin-V receptors. Additional integrins, α2β1, α3β1, and α5β1, also bind to the matrix proteins of the basalm lamina and provide the basal keratinocytes with essential information about their physical location. Detachment of integrins from their ligands may play a role in initiating differentiation (1, 2). Junction proteins in the basal keratinocytes include hemidesmosomal proteins, as well as desmogleins 2 and 3, desmocollin 3, E- and P-cadherins, and α1-connexin (34, 40, 44, 75). Many receptors have been demonstrated on the cell surface of the basal keratinocytes, providing responsiveness to the signals from surrounding cells (37, 41, 50, 51, 59, 64). In addition, basal keratinocytes can produce a large variety of secreted peptides, growth factors, cytokines, and chemokines (43, 47, 62). Thus the basal layer is not just a passive responder to signals from other cell types but can also be an initiator and modulator of the signals in the surrounding tissue.

The keratinocytes in suprabasal layers, spinosum, granulosum, and corneum, do not divide under normal conditions, but can be induced to do so, e.g., in wound healing (29). Their cytoskeleton contains keratins K1 and K10 and in upper layers K2c, in addition to the remnants of K5 and K14 from the basal layer. A new set of junctional proteins is produced, including desmocollin 1, plakophilin 1, and β2-connexin, along with desmogleins 1 and 3, E-cadherin, and occludin (34, 40, 44, 75), and so are the early differentiation markers, such as involucrin (19, 77). The cytoskeletal changes probably reflect the differences between the essentially immobile basal layer and the easily peripatetic suprabasal cells. These cells can also respond to a wide range of stimuli, but their responses are fundamentally different from the basal layer responses. For example, the testosterone action is mediated by the androgen receptor, while the other receptor, 5α-3β-reductase, is not expressed in the suprabasal cells (67). This report provides a comprehensive transcriptional profile of human epidermis, using the expression of β4 integrin as the discriminating factor, to comprehensively identify the differences in genes expressed in basal and differentiating layers of the epidermis, including the ECM components produced by the basal cells, the proteases in both the basal and suprabasal cells, and the lipid and steroid metabolism enzymes in suprabasal cells responsible for the permeability barrier. These differences are critical for the development and function of the epidermis, and understanding them could provide insights into the mechanisms of epidermal differentiation and its regulation.
αβ1 integrin is not a functional signal receptor in the suprabasal layers (2). The response to IL-1 represents another clear distinction between the basal and suprabasal keratinocytes; the former do not respond to IL-1, the latter, however, do. Suprabasal keratinocytes contain a functional IL-1R, whereas the basal layer expresses the “decoy,” inactive type II receptor (43). Interestingly, both respond to TNF-α, another proinflammatory signal (39, 41). A comprehensive examination of the signal transducing proteins and transcription factors in the epidermal layers has not been performed.

Although some of the proteins that characterize the epidermal layers have been described, a systematic analysis of layer-specific epidermal markers was not feasible because the necessary technology has not been available. An exciting technology to arise from the genome sequencing projects is DNA microarray hybridization, which provides a global view into changes of expression for a large set of genes. Using this methodology, we have presented systematic analyses of gene expression regulated by UV, interferon (IFN)-γ, and TNF-α in epidermal keratinocytes (4–6, 46).

To define comprehensively the genes expressed in different layers of human epidermis using microarrays, we have developed a new and simple procedure that yields large quantities of basal and suprabasal cells from human epidermis, based on the basal-specific expression of integrin-β4. This, coupled with the microarray technology, enabled us to establish the “transcriptome,” the comprehensive databank of genes expressed in the basal and suprabasal layers of the epidermis. We believe that these will open the door to much additional exploration of epidermal biology and pathology and will not only lead to a new, significantly deeper and more detailed understanding of the epidermis but also provide a rational base for more focused and effective therapies for skin diseases in the future. Furthermore, these studies pioneer analysis of the molecular processes of differentiation, a central problem of current molecular and cell biology.

MATERIALS AND METHODS

Keratinocyte culture. Normal epidermal keratinocytes from human foreskin were obtained from Dr. M. Simon (Living Skin Bank, Burn Unit SUNY Stony Brook, Stony Brook, NY). The cultures were initiated using 3T3 feeder layers as described (68) and then frozen in liquid N2 until used. Once thawed, the keratinocytes were grown without feeder cells in defined serum-free keratinocyte growth medium (KGM) supplemented with 0.05 mg/ml bovine pituitary extract, 5 ng/ml growth factor, and 1% penicillin-streptomycin (KGM from Gibco-BRL) at 37°C, in 5% CO2. The medium was replaced every 2 days. The cells were expanded through three passages for the experiments and trypsinized with 0.025% trypsin, which was added and the tissue filtered through Cell Strainer (Falcon). The trypsinization of the tissue was repeated twice more. The cells were collected by centrifugation, examined using trypan blue, and counted, and, if appropriate, the isolates were combined. This represented the unfractonated, total epidermal cell population (E).

Magnetic beads, M-450 anti-mouse IgG1, were prepared as suggested by the manufacturer (Dynal). The cells were incubated with the beads in the following ratio: 100 μl beads: 10–20 μg β4– antibody: 4 × 10⁶ cells (exactly) in 1× PBS, 0.1% BSA, at 4°C for 1–2 h. We used M-450 rat anti-mouse IgG1 beads and the 3E1 clone β4– antibody from Gibco-BRL. The beads were separated on a magnetic separator for 2–3 min and washed three or four times with PBS, collecting and combining the nonadherent, β4– cells as the suprabasal cell population (S). The beads bound to the β4+ cells, the basal cells (B), were used in RNA isolation without removing the cells from the beads.

Isolation of total RNA. To obtain RNA of appropriate quality for chip analysis from in vivo epidermis, we have tested several purification methods. After extensive experimentation, we settled on the following approach. First, the epidermal cells are disrupted, and the RNA is isolated with TRIzol (Gibco). This is followed by Qiashredders to homogenize cell extracts with centrifugation at 1,800 g for 2 min. DNA is removed with on-column DNase digestion using Qiagen RNases-free DNase Set. RNasey kits from Qiagen are used to prepare the RNA according to the manufacturer’s protocols. TRIzol gives good yields and effectively disrupts the epidermis, but the purity of the RNA is inadequate for the subsequent steps; the RNA isolation kit gives adequate purity but inefficiently disrupts the tissue, which is why the two are used in series. All solutions, starting with the one containing dispase, up to the final RNA elution, contain RNase inhibitor, because the skin is particularly rich in RNases, necessitating their inhibition. Five micrograms of total RNA are reverse transcribed, amplified, and labeled as described (30). Labeled cRNA is hybridized to oligonucleotide microarrays from Affymetrix. Arrays are washed, stained with antibiotin streptavidin/phycoerythrin-labeled antibody, and scanned using the GeneChip system (Hewlett-Packard) and GeneChip 3.0 software to determine the expression of each gene.

Northern and Western blot analyses. Approximately 5 μg of each RNA sample were loaded onto 1.5% agarose-formaldehyde gel. The RNA was transferred to a nylon membrane (Amersham) and cross-linked in a UV Stratalinker (Strategene). The Notch probe was synthesized using keratinocyte RNA and the RT-PCR kit (Promega) with the following primers GTTGATCGGCTCGGTAGTAA and ACACCCAGACGAGATGAC; the transglutaminase 1 probe with GCCCTAGCTACCTACGCA; and GCCACGTCTAGTCTTGGG; and the GAPDH probe with ACATAGCAAGGAGAAGATGAC; the 32P-labeled DNA microarray hybridization, which provides a global view into changes of expression for a large set of genes. Using this methodology, we have presented systematic analyses of gene expression regulated by UV, interferon (IFN)-γ, and TNF-α in epidermal keratinocytes (4–6, 46).
Fig. 1. Clean separation of the basal layer keratinocytes demonstrated on Western blots. Separated preparations of \( \beta^+ \) and \( \beta^- \) cells were tested with AE2 and AE3 antibodies, generous gifts from H. Sun. The same blot was stripped and reprobed with AE2. On the right, keratin proteins are identified. HDJ was used for loading control after stripping the involucrin antibody. Equivalent results were obtained in Northern blots using K5, K14, and K15 probes (not shown). Two independent separations from 2 individual donors gave identical results. Note that K5, basal keratin, is a stable protein that persists at the protein level in the suprabasal, \( \beta^- \) cells.

Fig. 2. Known differentially expressed functional categories: hemidesmosomal and cornified envelope components, cell cycle proteins and DNA replication enzymes. The genes preferentially expressed in the basal layer (B) are marked in red and have positive values for fold-differential expression; those overexpressed in the suprabasal layers (S) are marked with green color. The hierarchical clustering was performed using algorithms available at http://rana.stanford.edu/software (21).

Data from three individual donors were collected and analyzed both individually for each donor, as well as in aggregate for all three. The statistical analysis of the data included \( t \)-test and Mann-Whitney statistical evaluations. We calculated the medians of expression values for the each gene in the \( \beta^+ \), \( \beta^- \), and total epidermal cells, determined the expression ratios between \( \beta^+ \) and \( \beta^- \) cells, as well as between \( \beta^+ \) and total epidermal cells from the same donor, and then averaged these values for all three donors. We selected for further analysis the genes with at least one statistically significant difference in the \( t \)-test or Mann-Whitney analysis, and at least a twofold average difference of expression between either the \( \beta^+ \) vs. the \( \beta^- \), or the \( \beta^+ \) vs. the total epidermal cells.

We developed an extensive gene annotation table describing the molecular function and biological category of the genes present on the proteom.
chip (T. Banno and M. Blumenberg, unpublished). The table is primarily based on the data by J. M. Rouillard (70) and the Gene Ontology Consortium data http://dot.ped.med.umich.edu:2000/ourimage/pub/shared/JMR_pub_affyannot.html and http://cgap.nci.nih.gov/Genes/GOBrowser. The regulated genes were 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 (61).

The transcription factor binding sites in the regulated genes were identified with the oPOSSUM program (32). We first calibrated the parameters of the program using a set of identified NF-κB-regulated genes (5) to obtain the optimal statistical P values in the one-tailed Fisher exact probability analysis. We then used the same parameters for the differentially expressed genes. The comparison of the lists of differentially expressed genes in this study with those genes found to be regulated in epidermal keratinocytes by TNF-α or IFN-γ was performed using the LOLA set of programs (11).

Immunohistochemical staining of normal human skin. Pieces of normal human skin were obtained immediately after surgery, mounted in tissue Tec optimum cutting temperature compound (Sakura Finetek), and immediately frozen in liquid nitrogen. Sections, 4- to 6-μm thick, were obtained with a cryostat (Miles Laboratories). Slides containing frozen sections were dried and fixed in 100% acetone or 4% paraformaldehyde for 20 min at −20°C. After being blocked with 2% BSA in PBS, they were incubated with primary antibodies (Santa Cruz) at 4°C overnight. The sections were washed with PBS three times and incubated with secondary fluorescent-conjugated antibody IgG (Sigma-Aldrich) for 1 h at room temperature. After the final wash in PBS, they were mounted with media (Fluoromount-G, Southern Biotechnology Associates) and covered with coverslips. As a negative control, untreated skin was stained omitting the incubation with primary antibody. Stained sections were examined under a Carl Zeiss microscope, and digital images were collected with the Adobe Photoshop TWAIN_32 program.

RESULTS AND DISCUSSION

Separation of basal and suprabasal keratinocytes from human epidermis. We have developed a method for purification of basal layer keratinocytes in sufficient quantities and of appropriate quality for microarray analysis. The method consists of the following steps: 1) separation of the epidermis from dermis using dispase; 2) meticulous, fastidious, and gentle trypsinization of the epidermis to obtain undamaged single cells in suspension; 3) tagging the basal layer cells with β integrin antibody; and 4) separation of tagged cells using magnetic beads. We have considered and tried several approaches, with less success. For example, we found that fluorescence-activated cell sorting takes longer and damages cells more than the magnetic separation. We also found that α5 and β1 integrins are present at sufficient levels on suprabasal cells to preclude a clean separation from the basal layer (not shown).

The separation and purification of the epidermal layers were performed three separate times with samples from three separate donors. Reduction mammoplasty was chosen as the ideal source of skin because of the relatively narrow age range of donors (20s and early 30s), same sex, same body area, and a uniformly sun-protected site. The uniformity of the starting material can ensure the reliability and consistency of the results. However, the transcription profiles of skin from men, other body sites, age, and different sun exposure will probably turn out to have subtle but significant differences from those presented here.
To ascertain the purity of the separated cell populations, we used Western and Northern blots. As shown in Fig. 1, AE3 monoclonal antibody, specific for type II keratins (15, 60), detects K1, K2, K5, and K6 keratins in total epidermal cells and in the β4+ cells, whereas only the basal layer-specific K5 was found in the cultured and β4+ cells. AE2, which recognizes differentiation-specific keratins (16, 78), detects K1 and K10 only in total epidermis and β4+ cells, but not in cultured and β4+ cells. Similarly, the differentiation markers filaggrin and involucrin were found only in the β4+, but not in β4+, cells. These results demonstrate that the preparations of β4+ and β4− cells contain the appropriate markers that correspond to basal layer and nonbasal layer keratinocytes.

Microarrays of separated epidermal β4+ and β4− cells. The reliability of microarray analysis is considerably enhanced by multiple, redundant experiments, and these are commonly performed in triplicate. Therefore, we performed three separations of β4+ and β4− cells, from three different donors at three separate occasions, and tested each sample as in Fig. 1. From all three donors, we hybridized the three separate occasions, and tested each sample as in Fig. 1 performed in triplicate. Therefore, we performed three separations of β4+ and β4− cells, between and total epidermal cells from the same donor and then averaged these values. We selected the genes with at least one statistically significant difference in the cumulative analysis and at least a twofold average difference of expression between the β4+ cells and either the β4− or the total epidermal cells in the individual analysis. This selection identified 875 differentially expressed genes. The complete data are submitted to Gene Expression Omnibus repository (in process).

In these samples, the keratinocytes vastly outnumber all other cell types combined, by five- to tenfold. We do observe a low level of expression of melanocyte-specific markers, see below, but these do not affect significantly our results and conclusions.

Figure 2 contains three functional categories of genes known to be differentially expressed, and it serves as an independent control of our separation of the β4+ and β4− cells. The categories are hemidesmosomal genes, which are specific for basal cell and of which β4 was used for cell separation, cell cycle, and DNA replication genes, which are also specific for the basal, proliferative compartment of the epidermis and the epidermal differentiation and cornification markers, which are specific for the suprabasal, differentiating cells. As expected, the hemidesmosomal genes are expressed in the β4+ cells. We note that the “fold difference” for β4 integrin, the cell surface marker used to separate the β4+ and β4− cells, is 5.0. The well-recognized epidermal differentiation markers, filaggrin, involucrin, loricrin, and SPRRs, are overexpressed in the β4− cells. The S100A calcium-binding proteins are also considered to be epidermal differentiation markers because their genes bracket the epidermal differentiation complex (EDC) on chromosome 1, which contains the SPRR and LEP gene families and the filaggrin/involucrin/oricrin genes (22, 52, 55). Interestingly, here we show that the S100A genes are expressed in the basal cells, as has been reported previously (10, 22, 58, 69, 73). This suggests that the regulation of the EDC genes is more complex than believed hitherto.

Only basal cells replicate in healthy epidermis, and, accordingly, most cell cycle genes are found expressed in the β4+ cells, whereas the cell cycle inhibitors are in the β4− group. Similarly, the DNA replication enzymes are also in the β4+ cells; the two exceptions, POLB and NP, found expressed at higher levels in the β4−, play roles primarily in DNA repair and degradation. Taken together, the data in Fig. 2 agree very well with the expression studies of individual proteins (8, 20, 27) and explicitly validate our successful separation of epidermal keratinocytes from particular layers. This gave us confidence to examine several additional important and interesting functional groups.

Adhesion, ECM, and proteolysis. The basal and suprabasal cells express clearly distinct adhesion genes (Fig. 3). Desmosomal genes are expressed at higher levels in β4− cells, whereas all integrins are expressed in the β4+ cells. Interestingly, different adhesion markers are expressed in the different layers: the cadherins are in β4+ cells, conferring selective homotypic adhesion to the basal keratinocytes, whereas the

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Fig. 4. Metabolic enzymes, oncoproteins, and tumor suppressors. The suprabasal cells make the lipid and steroid metabolism enzymes. Basal cells make solute carrier gradient-driven transporters, whereas the suprabasal cells use ATP for transport. Note the oncoproteins expressed in the suprabasal and tumor suppressors in the basal cells.
tight- and gap-junction genes are expressed in β4− cells, contributing to the epidermal barrier. The ECM proteins are predominantly made by the β4+ cells, as expected because they abut the basement membrane. Laminin-V components (the ligand for β4 integrin), as well as collagen IV (a basement membrane component), and collagen VI (a matrix assembly scaffolding protein) are found expressed in the basal keratinocytes. These may play a role in maintenance of epidermal stem cells (57, 76) However, collagen I, which constitutes 80% of the dermal collagen, is absent, which leads us to believe that the contamination of fibroblasts in our preparation is negligible.

The β4+ and β4− cells produce distinct sets of proteases and their inhibitors (Fig. 3). Matrix metalloproteases MMP9 and MMP12 are produced by the β4+ cells, whereas the kallikreins are made by the β4− cells, reflecting different extracellular proteolytic targets of the two cells. While only the β4+ cells need and produce tissue inhibitor of metalloproteinases (SERPINs), each cell type produces a characteristic set of serine proteinase inhibitors (SERPINs).

The genes encoding extracellular proteins listed in Fig. 3 reflect the different milieus the two cell populations come into contact with. Although some of the markers were known to be differentially expressed in epidermal layers, we were surprised by several, e.g., by the extensive and diverse contribution to the basement membrane produced by the epidermis, by the specificity of the SERPINs produced by the different layers, and by the presence of cadherin 13 in the basal layer. These novel findings represent major advances in the understanding of epidermal structure and function and will inspire focused research in these areas.

**Metabolism.** Basal and differentiating layers of the epidermis have clearly different metabolic needs and processes and express different metabolic enzymes. In Fig. 4, we chose four
specific categories because of their special significance. The epidermal permeability barrier is formed by the differentiating cells in the granular layer and consists of a protein and a lipid component (23). Of the known genes involved in lipid metabolism, at least 11 are produced at higher levels in the cells. The appropriate steroid metabolism in the epidermis is also essential for the proper function of the cornified layer, evidenced by the fact that steroid sulfatase deficiency leads to X-linked ichthyosis (18). Consequently, we find six enzymes of the steroid metabolism preferentially expressed in the differentiating cells (Fig. 4). Thus, related to their barrier function, the suprabasal cells contain the enzymes for steroids and other lipids metabolism, which means that the basal cells only passively, without biochemical processing, transport the lipid barrier building blocks.

An interesting difference between the \( \beta^+ \) and \( \beta^- \) cells is found in the transporter protein genes: the \( \beta^+ \) and \( \beta^- \) cells express several solute carriers, which exchange ions with the extracellular medium, whereas the \( \beta^+ \) cells express ATPase family of transporters, which involve energy metabolism. Such characteristically different approaches to transport have not been described before and their further exploration may yield profound new understanding of the function of different epidermal layers.

We were particularly excited by the oncogenesis-related genes are differentially expressed in the \( \beta^+ \) and \( \beta^- \) cells. However, the details of these findings are not included in the provided text.
The differentially expressed oncogenes were found only in the β4−, whereas the tumor suppressors were in the β4+ cells. This may reflect the fact that only the basal cells can proliferate and are therefore more at risk for being transformed. We note that the list in the Fig. 4 is an abbreviated one; the oncogenes and tumor suppressors that are transcription factors or are involved in the cell cycle are presented in the respective figures.

**Cell surface receptors and secreted signaling proteins.** One of the essential protective functions of the epidermis is to alert the underlying tissues to the damage from the environment, as well as to respond to the stimuli from these tissues. In this as well, the epidermal layers have specific functions. Importantly, we find that both the basal and the suprabasal cells express genes for many cell surface receptors, as well as secreted signaling proteins, which means that they both respond, as well as signal to the surrounding tissue. The β4+ cells specifically express the decoy IL-1R to protect themselves from the IL-1 signal, and the FGF receptor (FGFR), which can respond to the proliferative signal of the keratinocyte growth factor and other FGFs (Fig. 5) (43, 49). In contrast, the β4− cells express the IL-1R-associated kinase, as well as receptors for thrombin, ephrin, etc. Interestingly, we find that the suprabasal cells specifically express the two relatives of the EGFR receptor (EGFR), ERBB2 and ERBB3. These have distinctive ligand recognition specicities, and ERBB2 dimerized with EGFR can be activated by neuregulin 2 (14, 24). Neuregulin 2 is differentially expressed by the β4+ cells (Fig. 5), which creates a possibility for paracrine signaling from the basal to the suprabasal cells. Conversely, we find FGF2 produced by the β4− cells, potentially signaling to the FGF-containing β4+ cells. Neither neuregulin 2 nor FGF2 was known previously to be expressed by keratinocytes!

The β4+ cells also express a relatively large number of genes encoding secreted proteins, i.e., growth factors and cytokines that target, in addition to epidermal cells, the underlying fibroblasts and endothelial cells, perhaps lymphocytes as well. Prominent among these are the members of the transforming growth factor (TGF)-β family. We find that the β4− cells selectively express higher levels of apolipoproteins D and E, although the basal cells were shown to secrete ApoE (7).

Our data identify two potential paracrine signaling mechanisms between the basal and suprabasal cells, an area of research that has not been intensely addressed in the past. These are the neuregulin/ERBB and FGF2/FGFR pairings. The identification of the receptors in different epidermal layers suggests a possibility for targeted differential therapy of proliferative diseases, both to enhance proliferation, e.g., in chronic wounds, and to inhibit it, e.g., in psoriasis.

We were particularly intrigued by the production of neuregulin 2 and its co-receptor ERBB2 in the basal and suprabasal cells, respectively. Their roles in skin cancers have not been fully described, and the discovery of this paracrine pathway correlates with the predominantly suprabasal expression of oncogenes and the basal expression of tumor suppressors. We suspect that the abundance of cell cycle and DNA replication genes makes the basal cells particularly susceptible to oncogenic transformation. The data presented here can form a basis of the analysis of genetic changes necessary for the occurrence of basal and squamous cell carcinomas.

To confirm that some of the microarray findings accurately reflect the differential expression in human epidermis in vivo, we used immunohistochemistry on skin sections (Fig. 6). We tested the expression of IGFBP3, a marker of basal β4+ cells, and NOTCH3, a marker of suprabasal, β4− cells. We find that the in vivo data perfectly correspond to the expectations from the microarray experiments, thus validating our results.

**Differentially expressed signal transduction proteins and transcription factors.** The differences in the signaling pathways in the two cell types are very interesting (Fig. 7). For example, the Ras pathway genes are mainly found expressed in the β4+ cells, perhaps reflecting their proliferative capability. Playing an important role in proliferation, the Ras family may regulate the cell cycle and DNA replication. Interestingly, the diacylglycerol, inositol phosphate, and prostaglandin pathways, which means that they both respond, as well as signal to the surrounding tissue. The β4+ cells specifically express the decoy IL-1R to protect themselves from the IL-1 signal, and the FGF receptor (FGFR), which can respond to the proliferative signal of the keratinocyte growth factor and other FGFs (Fig. 5) (43, 49). In contrast, the β4− cells express the IL-1R-associated kinase, as well as receptors for thrombin, ephrin, etc. Interestingly, we find that the suprabasal cells specifically express the two relatives of the EGFR receptor (EGFR), ERBB2 and ERBB3. These have distinctive ligand recognition specificities, and ERBB2 dimerized with EGFR can be activated by neuregulin 2 (14, 24). Neuregulin 2 is differentially expressed by the β4+ cells (Fig. 5), which creates a possibility for paracrine signaling from the basal to the suprabasal cells. Conversely, we find FGF2 produced by the β4− cells, potentially signaling to the FGF-containing β4+ cells. Neither neuregulin 2 nor FGF2 was known previously to be expressed by keratinocytes!

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lipid metabolism in the process of barrier formation. Various protein kinases and phosphatases are differentially expressed in the different epidermal layers, and we note their abundance in protein kinases and phosphatases are differentially expressed in lipid metabolism in the process of barrier formation. Various to a cornified layer, are highly responsive to signaling.

We note that the β4+ and β4− cells express Jagged 2 and Notch 3, respectively, potentially establishing another juxta-crine signaling pathway. Notch signaling regulates epidermal differentiation (63), although these two particular proteins were not known to be expressed in epidermis. It will be interesting to ascertain whether the Jagged 2 and Notch 3 expressing cells are juxtaposed in the epidermis.

Similarly instructive are the differences in the transcription factors expressed in the basal and suprabasal cells, listed in Fig. 8. We do not know enough to assign a function to each of the differentially expressed gene in this functional category, but we believe that the field will be able to fill this gap.

The SMAD genes are well represented in the β4+ fraction, reflecting the specific effects of TGF-β on the basal cells (26, 37, 79). We were intrigued by the TGF-β-responsive machinery specific for the basal cells. This correlates well with the previous results from our and other laboratories, suggesting that TGF-β promotes the basal cell phenotype in skin (37). STAT5, responsive to IL-3, prolactin, etc., is also preferentially found in the β4+ cells. CCAAT enhancer-binding protein-β is found in the β4− cells, confirming its role in epidermal differentiation (17, 72); interestingly, we find NFKBIE, an activator of NF-κB in the β4+ and TANK, an inhibitor of NF-κB, in the β4− cells. Several IFN-α and TNF-α-responsive transcription factors are present in the β4+ population, demonstrating that the suprabasal cells are clearly competent to respond specifically to proinflammatory and immunomodulatory signals.

The β4+ and β4− cells contain characteristic sets of zinc finger, Ets proteins, and nuclear receptors with associated co-regulators. Interestingly, we do not find basosinulin differentially expressed, presumably because significant levels of this protein are made both in the early suprabasal keratinocytes and in the basal cells (74). SREBF2, a sterol regulatory factor, is preferentially expressed in the β4− cells (DLX3 is not represented on the microarrays). DLX5 may play an essential role in regulating epidermal differentiation. Parenthetically, IRX5, a member of the Iroquois family, is probably expressed in melanocytes, see below.

Melanocyte-derived transcripts. Although keratinocytes are by far the predominant cell type in the epidermis, melanocytes constitute a significant minority; it is therefore not surprising that several melanogenesis enzymes have been detected by the arrays (Fig. 9). Melanocytes do not express β4 integrin and are found in the β4− population. We noticed a peculiarity in the expression of melanogenesis enzymes: they were expressed at significantly higher levels in the β4− population than in the unseparated, total epidermal cells. For a great majority of genes, the expression levels in the β4− population and in the total epidermal cells are very similar because the β4− cells are
the predominant population in the epidermis. The overrepresentation of melanocytes in the $H9252$ vs. $H11002$ cells population derives, we believe, from the isolation procedure: being in the basal layer, the melanocytes are quantitatively released by trypsinization, whereas many of the $H9252$ keratinocytes, especially those at advanced stages of differentiation, are more resistant to trypsinization, remain in undigested cell aggregates, and are lost from the $H9252$ isolates. The skewed $H9252$ vs. total cells ratio of melanogenic genes led us to ask whether there are additional genes with similarly skewed ratios, i.e., “expression signatures.” Using a twofold cutoff for the $H9252$ vs. total epidermis expression ratio, we found 49 such genes (Fig. 9). Among these is vimentin, a known melanocyte cytoskeletal component, which validated our approach. We note that for all these genes there is a statistically significant difference in expression between the $H9252$ and $H11002$ cells, which is not necessarily true for the $H9252$ vs. total cells comparisons (Fig. 9, columns 4–7).
tional genes, encoding cytoskeletal, metabolic, signaling proteins, and transcription factors, as well as nine uncharacterized expressed sequence tags (ESTs), none previously known to be expressed in melanocytes. We are particularly intrigued by the relatively high levels of c-Myc, an oncogene important in melanomas. These data will undoubtedly be useful to studies of melanocytes, which, to date have been largely restricted to melanoma cells. Parenthetically, the chromosomal locations of the melanocyte markers appear random, i.e., we did not detect clusters of melanocyte genes, similar to the epidermal differentiation gene clusters.

These genes, presumably, derive from melanocytes, although we cannot at present exclude the possibility that they derive from other cell types that reside in the basal layer, such as Langerhans or Merkel cells. We find no fibroblast markers, such as Coll1, at all in our microarrays, and therefore we believe that the contributions of dermal cells, fibroblasts, endothelial cells, and smooth muscle cells, are below detection levels.

Keratinization markers. Although the melanocyte markers are overrepresented in the β+- population relative to the total epidermal cells, another group of genes was underrepresented in the β4+- population relative to the total epidermal cells. Careful examination of the functions of genes with this characteristic expression signature pointed to the known cornified envelope proteins, markers of advanced epidermal differentiation. These genes are, of course, not expressed in the β4+ cells and, relative to the total epidermis, are selectively missing from the β4+- population as well. We believe that the cells in the most advanced stages of differentiation are more resistant to trypsinization, remain in clusters, and are lost during the purification of the β4+-cells, whereas the harsh RNA purification procedure releases the mRNA equally from all cells of the total epidermal preparations, including the cells in the most advanced stages of cornification. Alternatively, the mRNA for markers of advanced differentiation may be selectively degraded during the isolation procedure, targeted by the epidermal RNases.

Using a 1.7-fold cutoff for the total epidermis/β4- expression ratio, we find 83 differentially expressed genes (Fig. 10). Among these are transglutaminases, loricrin, involucrin, and filaggrin, well-known markers of cornification (8, 20, 27). We also find a significant number of lipid and steroid metabolic enzymes, kallikreins, etc. In this group we find ARS, the gene that, when mutated, causes Mal de Maleda, an inherited disorder of cornification (3, 25, 53). In addition, proteases and protease inhibitors are amply represented among these genes, reflecting the extensive remodeling of the stratum corneum on one hand and protection from the environmental microorganisms on the other. Similarly, in this group we find claudin and occludin, the tight junction proteins with important functions in permeability barrier and antimicrobial defense (4, 31, 36).

Several interesting regulatory genes fall in this group, encoding protein kinases and phosphatases, and we would like to draw attention to Fyn, which may play a role in regulation of epidermal differentiation (12, 33).

Using this expression signature, we identified a much larger set of potential late epidermal differentiation markers, many of which we were not aware of before. The newly identified genes include signaling proteins and transcription factors, potentially regulators of the final steps in keratinocyte terminal differentiation. Interestingly, the location of these genes in the human genome is not random. As mentioned above, many cornified envelope proteins are encoded by the EDC on Ch1q21, and indeed, we find a cluster of seven genes mapping at that locus. When we examined the genomic positions of all genes in Fig. 10, we identified additional chromosomal bands with multiple differentiation specific genes, 6p21, 11q13, and 19q13. We hypothesize that these clusters also have functional significance, similar to the already described cluster on 1q21. We are pursuing these leads in the hope of identifying additional EDCs and associating them with mapped human inherited skin diseases.

We would like to point out that, due to space limitations, we have not included in the above analysis genes encoding several important functional categories, e.g., cytoskeletal and membrane proteins, apoptosis, immune response, and RNA metabolism; all these can be found in the Supplement. The online version of this article contains supplemental data.

Comparison of the lists of differentially expressed genes with their assigned ontology functions confirms the above analysis (Fig. 11). This is particularly apparent in the

![Fig. 11. Ontological categories of differentially expressed genes. Biological processes (left) and cellular components (right) overrepresented among the genes with β4+; β4-; upper layer cells and melanocyte signature expression. The P value shows the probability that the specific process or component is as overrepresented in a same-size list of random genes. Only categories with P value <10^-3 are shown. Note the redundancy among similar categories. No cellular component was found to be overrepresented among the genes with the melanocyte-characteristic expression signature.](http://physiolgenomics.physiology.org/)
cellular component categories, where in the basal cells the ECM and basement membrane genes are overrepresented, whereas the intercellular junction components are characteristic of the β4− cells and the cornified envelopes in the cells of the “upper layer.” The biological processes characteristic of the β4+ cells include transport of nutrients and cell motility. In contrast, the β4− cells’ epidermis development, keratinocyte differentiation, eicosanoid, lipid, and steroid biosynthesis are overrepresented. These biological processes are even more pronounced in the terminally differentiating, upper layer cells. Melanin biosynthesis and pigmentation are, as expected, greatly overrepresented among the melanocyte signature genes.

When we compared the lists of differentially expressed genes in the β4+ and β4− cells with the keratinocyte genes regulated by IFN-γ or TNF-α, we found several interesting correlations. Both IFN-γ and TNF-α induced β4+, basal layer-specific genes ANXA3, C1R, C1S, ITIF3, ITGB4, LAMA3, LAMB3, MMP9, and NFKBIE. These are associated with the basement membrane and the proinflammatory processes. Both IFN-γ and TNF-α induced β4−-specific genes CST6, GPR109B, PTPN12, and QPCT, and TNF-α induced but IFN-γ suppressed differentiation-specific markers FLG, IVL, and SERPINB3. This result confirms our suggestion that IFN-γ suppresses epidermal differentiation. Furthermore, IFN-γ, but not TNF-α, induced β4− genes CLDN4 and OCLN, encoding tight junction proteins, presumably to build up tight junctions and prevent paracellular transepidermal conduct of viruses, as we suggested previously (3).

The data presented here define the transcriptional changes that occur as the epidermal keratinocytes leave the basal layer and commence differentiation. Specifically, we find the genes encoding cell cycle and DNA synthesis proteins expressed in the basal keratinocytes, whereas the cornified envelope proteins and other differentiation markers are expressed in the suprabasal ones. This is as expected and verifies the separation of the layers using our new methodology. But more than that, the data identify and define the fundamental biological differences between the progenitor and the operational cells of the epidermis.

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