ORIGINAL ARTICLE

Epigenetic Modulation of Gene Expression during Keratinocyte Differentiation

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Background: Epigenetic modulation of gene expression occurs by various methods, including DNA methylation and histone modification. DNA methylation of specific genes may affect the chromatin structure, preventing access by the transcriptional machinery. Although gene expression is dramatically changed during keratinocyte differentiation, there is no evidence of epigenetic modulation during the process of epidermal stratification. **Objective:** We investigated whether epigenetic modulation is involved in keratinocyte differentiation-specific gene regulation. **Methods:** We used trypsin to produce epidermal fragmentation (named T1-T4) and performed a morphological analysis using hematoxylin-eosin stain and cytokeratin expression based on reverse transcription polymerase chain reaction. We then constructed a DNA methylation microarray. **Results:** Each epidermal fragment showed morphological features of the epithelial layer. T1 represented the basal layer, T2 was the spinous layer, T3 was the granular layer, and T4 was the cornified layer. The level of the K14 proliferation marker was increased in the T1 fraction, and the level of K10 differentiation marker was increased in the T2-T4 fractions. Using a methylation microarray with the T1 and T4 fractions, we obtained many hypermethylated and hypomethylated genes from differentiated keratinocytes. **Conclusion:** The importance of epigenetic modulation in target gene expression during keratinocyte differentiation is identified. **(Ann Dermatol 24(3) 261~266, 2012)**

-Keywords-
Cell differentiation, DNA methylation, Epigenomics, Keratinocytes

INTRODUCTION

Keratinocytes progress through the suprabasal layers during epidermal differentiation, undergoing complex and tightly regulated biochemical modification leading to cornification and desquamation. The development of DNA chips in combination with in vitro models of human epidermal morphogenesis enhances our ability to identify all genes that are involved in the process of epidermal stratification[1].

Epigenetics is the study of potentially heritable phenotypical differences in the absence of variation in the genetic code. That genetic variability is important in the etiology of many diseases is well established. However, environmental factors, including diet and lifestyle, modulate that susceptibility in part through epigenetic changes[2]. Epigenetic phenomena involve both intracellular and intercellular interactions, leading to alterations in reversible phenomena such as cell signaling and DNA modification[3]. Epigenetics is important in the pathogenesis of many skin diseases[4]. For common skin cancers, aberrant methylation of tumor suppressor gene promoters is associated with their transcriptional inactivation[5]. Hypomethylation is associated with activation of systemic autoimmune diseases, such as systemic lupus erythematosus, and scleroderma[6].

Epigenetic factors may also play a role in the pathogenesis of psoriasis and other inflammatory skin diseases[7]. However, there are no reports that identify an epigenetic modulation of gene expression during keratinocyte differentiation. We investigated whether epigenetic modulation is involved in keratinocyte differentiation-specific gene regulation.

MATERIALS AND METHODS

Skin samples and preparation

Normal human skin samples were obtained from circumcisions under the written informed consent of the donors, in accordance with a process approved by the Ethical Committee and Institutional Review Board of Chungnam National University Hospital (cnuh 2011-06-104). Subcutaneous fat was promptly removed and strips of skin were incubated, epidermis side up, for 1 hour at 4^oC in phosphate-buffered saline (PBS) containing 0.5 mg/ml of thermolysin (Sigma, St Louis, MO, USA). The epidermis was dissected free from the dermis using forceps, and rinsed in cold PBS. Epidermal fragments were either immediately frozen for total RNA extraction, or incubated in a 1× trypsin- ethylenediaminetetraacetic acid (EDTA) solution (Invitrogen, Carlsbad, CA, USA) at 4^oC under gentle agitation for 15 minutes. The remaining epidermal fragments were rinsed in cold PBS and incubated in another trypsin-EDTA solution. Fetal calf serum (Invitrogen) was added to the suspended cells (10% final concentration). After centrifugation, the cells were frozen as dry pellets. The procedure was repeated twice, leading to three successive fractions of dissociated cells named the T1, T2, and T3 fractions, and a residual fragment named the T4 fraction.

Morphological analysis of epidermal samples

After each trypsin incubation, an aliquot of epidermal fragments was fixed and embedded in paraffin, and sections (10 μm) were stained with H&E.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from epidermal fragments using an Easyblue RNA extraction kit (Intron, Daejeon, Korea) according to the manufacturer's recommended protocol. Two μg of total RNAs was reverse transcribed using moloney-murine leukaemia virus reverse transcriptase (ELPIS Biotech, Daejeon, Korea). Aliquots of an RT mixture were subjected to PCR cycles with a specific primer set. All primers used in this study were designed using the Primer 3 input (http://frodo.wi.mit.edu/primer3/) and are as follows: keratin 14 (K14) 5′-CAGTTCACCTCCT

CCAGCTC and 5′-TCCTCAGGTCCTCAATGGTC, keratin 10 (K10) 5′-CTACTCTTCCTCCCGCAGTG and 5′-TTGCC ATGCTTTTCATACCA, cyclophilin 5′-CTCCTTTGAGCTG TTTGCAG and 5′-CACCACATGCTTGCCATCCA. PCR fragments were electrophoresed on 1.0% agarose gel and subsequently visualized using ethidium bromide staining.

Methylated-CpG island recovery assay-assisted microarray analysis

Genomic DNA was isolated from the T1 and T4 fractions using a DNeasy purification kit (Qiagen, Valencia, CA, USA). We sonicated genomic DNA to produce random fragments ranging in size from 100 to 600 bp. Two Microgram of MBD2bt protein was incubated with 1 μg of sonicated genomic DNA in a binding reaction mixture (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 3 mM MgCl2, 0.1% Triton-X100, 5% glycerol and 25 mg/ml of bovine serum albumin) for 4 hours at 4^oC on a rocking platform. The DNA-protein complex was precipitated using preblocked nickel-magnetic beads. After washing the pelleted magnetic beads five times in a washing buffer (binding buffer containing 700 mM NaCl), the methylated DNA-enriched DNA fraction was purified using a Qiaquick PCR purification kit (Qiagen).

Enriched methyl DNA was amplified using a whole genome amplification kit (GenomePlex® Complete Whole Genome Amplification Kit, Sigma) as recommended by the manufacturer. A second round amplification was performed using an aminoally-dUTP mixture (Sigma) and a whole genome amplification kit. The amplified products were labeled by coupling with Cy3 and Cy5-monofunctional dye (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Then, dye-labeled DNA samples were purified and quantified using an ND-1000 spectrophotometer (Nano-Drop Technologies, Inc., Wilmington, DE, USA).

After checking the labeling efficiency, each 2.5 to 5 μg of Cy3-labeled and Cy5-labeled DNA target was mixed, and then resuspended in a 2X hybridization buffer with Cot-1 DNA, an Agilent 10X blocking agent, and de-ionized formamide. The arrays were hybridized at 67^oC for 40 h using an Agilent Hybridization oven (Agilent Technology, Santa Clara, CA, USA).

Data acquisition and analysis

Hybridization images were analyzed using an Agilent DNA Microarray Scanner (Agilent Technology) and data quantification was performed using Agilent Feature Extraction software (Agilent Technology). Preprocessing of raw data and normalization steps were performed using GeneSpring 7.3.1 (Agilent Technology). The individual CpG methylation difference was directly compared based

on ratios of signals from control sample DNA.

RESULTS

We first applied trypsin to epidermal fragments. Repetitive incubation of human epidermal pieces with trypsin was performed to achieve four suspended cell fractions. Morphological analyses revealed that the T1 fragments were mostly composed of the basal layer, T2 was the spinous layer, and T3 was both the spinous and granular layers. Residual epidermal fragments were mostly composed of the cornified layer (Fig. 1). RT-PCR was used to assess the relative cytokine expression levels in the successive cell fractions. K14 was used as a basal proliferation marker, and K10 as a keratinocyte differentiation marker. Cyclophilin was used to compare total amounts as an internal control. The level of K14 was increased in the T1 fraction, and the level of K10 was increased in the T2, T3, and T4 fractions (Fig. 2). The T1 fraction represented the basal and proliferative layers, while the T2, T3, T4 fractions represented the suprabasal and the differentiation layers.

To investigate epigenetic modulation of gene expression during keratinocyte differentiation, we isolated genomic DNA from the T1 and T4 fractions and applied this DNA to a methylation microarray chip. After hybridization, the
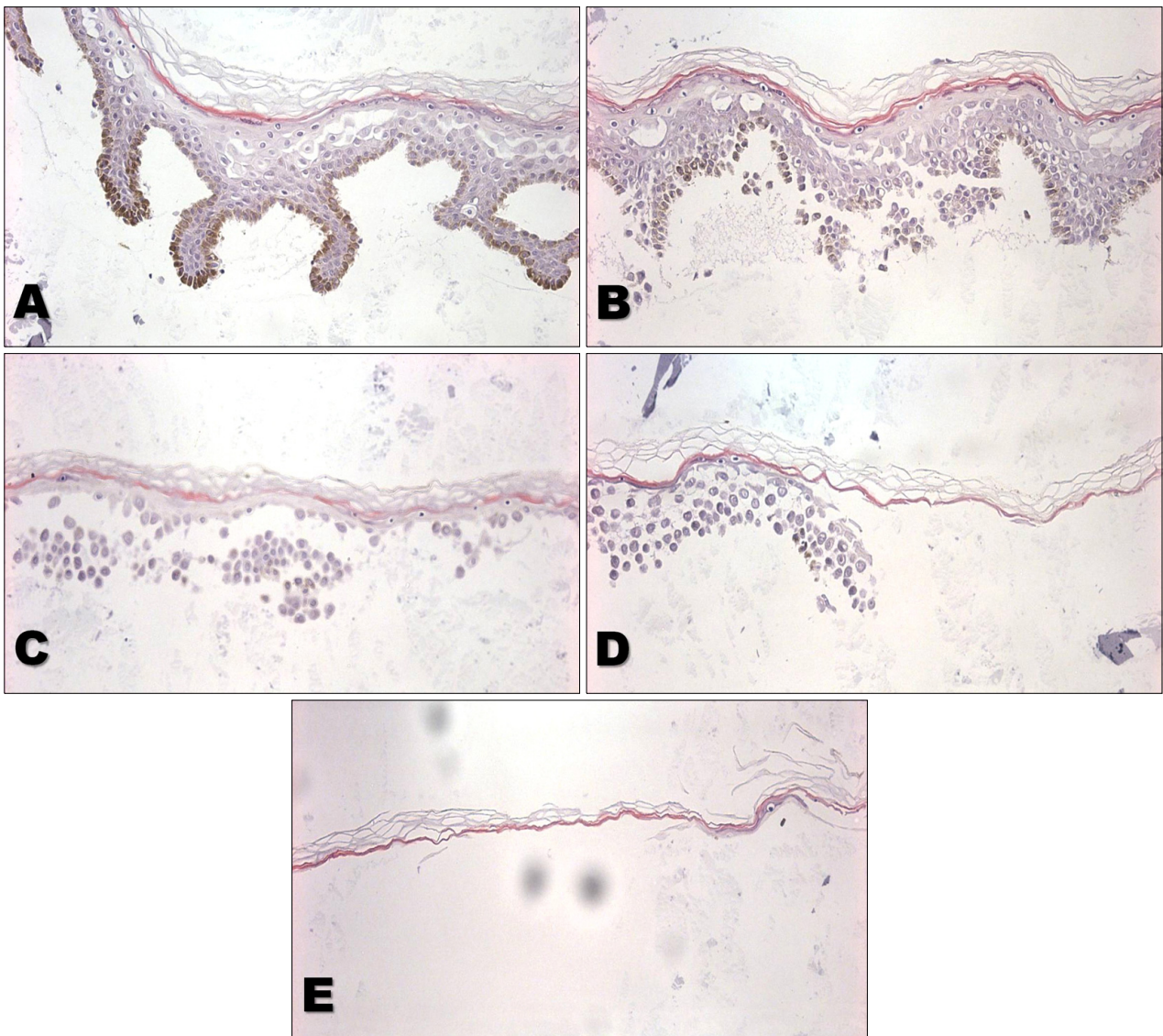

Fig. 1. Histologic analysis of an epidermal sample. (A) A H&E stained section of the entire epidermis after thermolysin incubation and removal of the dermis. Epidermal fragments remaining after the first, second, and third trypsin treatments, respectively, constitute the T1 (B), T2 (C), and T3 fractions (D). Fragments shown in (E) are mainly composed of the cornified layer and constitute the T4 fraction.

microarray slide was scanned and analyzed. Each gene was spotted according to its signal intensity as T1 (Cy3) versus T4 (Cy5). Graphs are shown (Fig. 3) on a log scale using DNA methylation microarray data.

Labeled DNA samples were hybridized to human CpG island microarrays containing 237,000 oligonucleotide probes covering 27,800 CpG islands. To select multiple probes for an enriched genes test, methylation candidate genes were chosen when their probes showed ≥ 2.0-fold in the methylation test, compared with control samples in at least two adjacent probes, allowing a one-probe gap
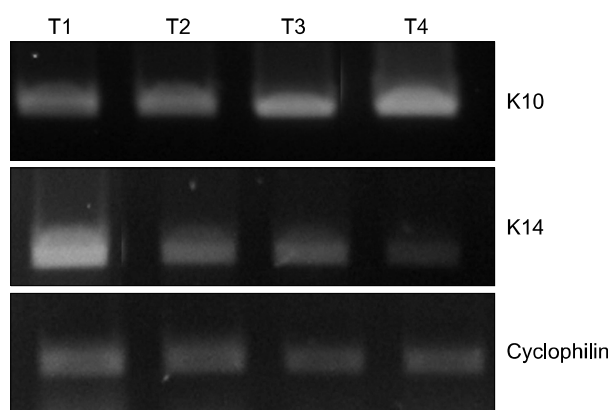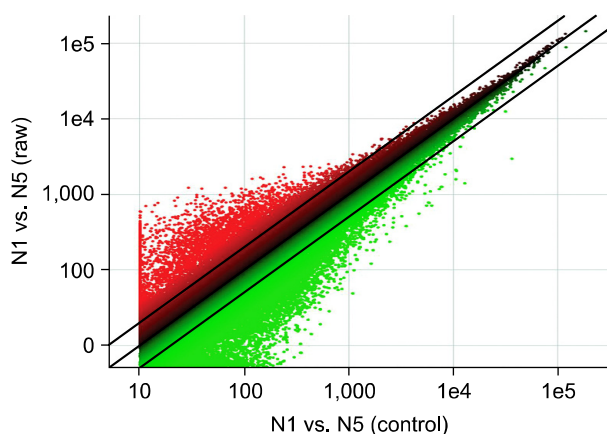

Fig. 2. Reverse transcription polymerase chain reaction analysis for K14 and K10 in an epidermal sample. The level of the epidermal differentiation marker keratin 10 (K10) is increased, while the level of the proliferation marker keratin 14 (K14) is decreased in the fractionated epidermal cells, indicating sample preparation was successfully performed.

within the CpG islands. We examined the hypermethylation status of these 9 genes: PCDHGA3, FBXL17, SPG20, DUS3L, MRPL36-NDUFS6, PRKD3, PSCD2, C2orf3, FLJ32447 and the hypomethylation status of these 9 genes: ZZEF1, ZNHIT3, ZFAND2A, ZBTB11, YWHAQ, WDR54, VAX1, TXNDC9-EIF5B, and SPCS2. Among these genes, PSCD2 was the most significant gene in hypermethylation field, and YWHAQ was the most reliable gene in hypomethylation field. Detailed information is shown in Table 1.

DISCUSSION

As the keratinocyte differentiation process occurs along a pathway that leads to cell cycle arrest and terminal differentiation, a complex program of gene expression must be coordinated[1,8]. Many differentiation-related genes, including those encoding transglutaminases 1 and 3, involucrin, cornifin, loricrin, filaggrin, and small proline-rich proteins, are expressed in a temporally regulated manner[9,10]. New genes such as Brn2[11], Nkx 2.5[12], plasminogen activator inhibitor-2[13] has been suggested to play a role in keratinocyte differentiation.

There are three major molecular mechanisms mediating epigenetic change. They are DNA methylation, Histone modification, and MicroRNA interference[4]. Methylation of the cytosine and guanine dinucleotides (CpG islands) occurs in the promoter region of approximately 40% of genes in higher eukaryotes. Methylation usually represses gene transcription[14], While histone acetylation is generally
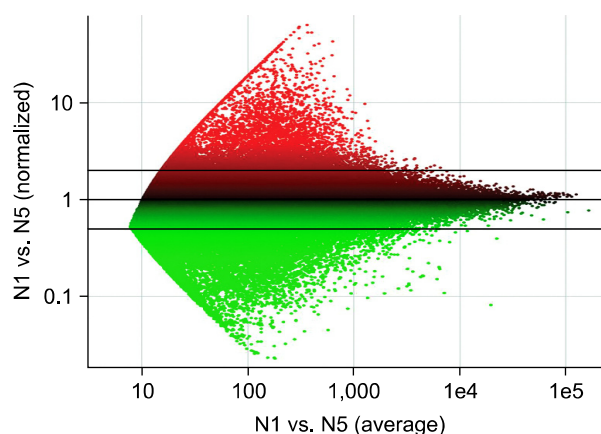
X-axis: 20090409 (default interpretation), N1 vs. N5
Y-axis: 20090409 (default interpretation), N1 vs. N5
Colored by. 20090409 (default interpretation)
Gene list: all genes (237220)

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Y-axis: 20090409 (default interpretation), N1 vs. N5
Colored by. 20090409 (default interpretation)
Gene list: all genes (237220)

Fig. 3. Scatter plot of the methylation microarray. Genomic DNA was isolated from the T1 and T4 fractions, labeled with Cy3 and Cy5, then applied to the methylation microarray chip. After hybridization, the microarray slide was scanned and analyzed. Each gene was spotted according to its signal intensity.

Table 1. DNA methylation microarray data. Hypermethylated genes (A) and hypomethylated genes (B) from differentiated keratinocytes

Gene name	Symbol	Accession number	Map
(A)			
Protocadherin gamma subfamily A, 3	PCDHGA3	NM_018916	5q31
F-box and leucine-rich repeat protein 17	FBXL17	NM_022824	5q21.3
Spastic paraplegia 20, spartin (Troyer syndrome)	SPG20	NM_015087	13q13.3
Dihydrouridine synthase 3-like (*S. cerevisiae*)	DUS3L	NM_020175	19p13.3
Mitochondrial ribosomal protein L36	MRPL36-NDUFS6	NM_032479	5p15.3
Protein kinase D3	PRKD3	NM_005813	2p21
Pleckstrin homology, Sec7 and coiled-coil domains 2 (cytohesin-2)	PSCD2	NM_017457	19q13.3
Chromosome 2 open reading frame 3	C2orf3	NM_003203	2p11.2-p11.1
Hypothetical protein LOC151278	FLJ32447	NM_153038	2q36.1
(B)			
Zinc finger, ZZ-type with EF-hand Domain 1	ZZEF1	NM_015113	17p13.2
Zinc finger, HIT type 3	ZNHIT3	NM_001033577	17q12
Zinc finger, AN1-type domain 2A	ZFAND2A	NM_182491	7p22.3
Zinc finger and BTB domain containing 11	ZBTB11	NM_014415	3q12.3
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide	YWHAQ	NM_006826	2p25.1
WD repeat domain 54	WDR54	NM_032118	2p13.1
Ventral anterior homeobox 1	VAX1	NM_199131	10q26.1
Thioredoxin domain containing 9	TXNDC9-EIF5B	NM_005783	2q11.2
Signal peptidase complex subunit 2 homolog (*S. cerevisiae*)	SPCS2	NM_014752	11q13.4

S. cerevisiae: *Saccharomyces cerevisiae*.

linked to activation of transcription[15]. Another mechanism is the effect of nonprotein coding mRNAs on the transcription of other genes and protein synthesis[16]. The microRNA pathways may be more promising as therapeutic targets, as their effects are more specific than methylation or histone modification.

Knowledge of the contribution of epigenetic mechanisms to the pathogenesis of skin disease has expanded considerably over the last few years, particularly in the field of skin cancer and inflammatory skin diseases[9,17]. Despite intensive research into skin disease, epigenetic modulation during keratinocyte differentiation is not yet understood.

We used trypsin for epidermal fragmentation, as described previously[18] with successive short-term enzyme incubation to progressively detach cells from the deep layers, and to purify the cells. Incubations were performed at 4°C to stop cellular metabolic activity and to preserve the mRNA pool from degradation. This point is crucial as many growth factors, cell cycle regulators, and transcription factors are encoded by short-lived mRNAs.

We performed methylation DNA microarray analysis with genomic DNA isolated from the basal (T1) and cornified layers (T4). A Methylated-CpG assisted microarray analysis was performed as described previously[19]. We examined 9 hypermethylated genes and 9 hypomethylated genes. Most of these genes had not previously been associated with keratinocyte differentiation. PSCD2 (pleckstrin homology, Sec7 and coiled-coil domain 2) was the most significant gene in hypermethylation field, and YWHAQ (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide) was the most reliable gene in hypomethylation field.

The PSCD2 gene functions to promote activation of adenosin diphosphate-ribosylation factor (ARF) through replacement of GDP with GTP. Members of this family have an identical structural organization that consists of an N-terminal coiled-coil motif, a central Sec7 domain, and a C-terminal pleckstrin homology domain[20]. The functions of this family include mediating regulation of protein sorting and membrane trafficking[20,21]. Although there are reports of a regulatory role in development of neuronal processes[22], the role of PSCD2 during keratinocyte differentiation has not yet been investigated.

YWHAQ is a gene associated with an adapter protein that is implicated in regulation of a signaling pathway. Binding generally results in modulation of the activity of the binding partner (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide)[23,24]. YWHAQ is directly involved in cellular processes, such as cytokinesis, cell-contact inhibition, anchorage-independent growth, and cell adhesion, processes that often become deregulated in diseases like cancer[24,25]. Recently, there was a report that YWHAZ, an isoform of YWHAQ,

significantly suppressed the growth rate of head and neck squamous cell carcinoma cell lines, and overexpression of YWHAZ in human keratinocytes promotes overgrowth and morphological changes[26,27].

We have identified many hypermethylated and hypomethylated genes from differentiated keratinocytes that are involved in epigenetic regulation of keratinocyte differentiation. As this is a preliminary study, more work is necessary to determine whether changes in the methylation status of these candidate genes actually control keratinocyte differentiation. Although extensive work in this field is clearly needed, our preliminary findings highlight the importance of epigenetic modulation in keratinocyte differentiation-specific gene regulation. Furthermore, we provide useful information for future development of novel therapeutic and preventive tools for many skin diseases associated with abnormalities in keratinocyte differentiation.

ACKNOWLEDGMENT

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