Protein-coding and non-coding gene expression analysis in differentiating human keratinocytes using a three-dimensional epidermal equivalent

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Running Title: mRNA, miRNA and long ncRNA expression in epidermal keratinocytes

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Abstract

The epidermal compartment is complex and organized into several strata composed of keratinocytes (KCs), including basal, spinous, granular, and cornified layers. The continuous process of self-renewal and barrier formation is dependent on a homeostatic balance achieved amongst KCs involving proliferation, differentiation, and cell death. To determine genes responsible for initiating and maintaining a cornified epidermis, organotypic cultures comprised entirely of stratified KCs creating epidermal equivalents (EE) were raised from a submerged state to an air/liquid (A/L) interface. Compared to the array profile of submerged cultures containing KCs predominantly in a proliferative (relatively undifferentiated) state, EEs raised to an A/L interface displayed a remarkably consistent and distinct profile of mRNAs. Cultures lifted to an A/L interface triggered the induction of gene groups that regulate proliferation, differentiation, and cell death. Next, differentially expressed microRNAs (miRNAs) and long non-coding (lncRNA) RNAs were identified in EEs. Several differentially expressed miRNAs were validated by qRT-PCR and Northern blots. miRNAs 203, 205 and Let-7b were up-regulated at early time points (6 hrs, 18 hrs and 24 hrs) but down-regulated by 120 hrs. To study the lncRNA regulation in EEs, we profiled lncRNA expression by microarray and validated the results by qRT-PCR. Although the differential expression of several lncRNAs is suggestive of a role in epidermal differentiation, their biological functions remain to be elucidated. The current studies lay the foundation for relevant model systems to address such fundamentally important biological aspects of epidermal structure and function in normal and diseased human skin.

Key Words: Keratinocytes; differentiation; mRNA, transcriptome, miRNA

Introduction

Human epidermis, the outermost protective coat of the body, can be morphologically divided into four distinct strata: basal, spinosum, granulosum, and corneum. Epidermal keratinocytes (KCs) undergo a series of complex morphological changes during their maturation into anucleated corneocytes that are responsible for creating and maturating the barrier function of skin (Eckert et al. 1997). The continuous process of epidermopoiesis is dependent on a homeostatic balance achieved amongst KCs involving proliferation, differentiation, and cell death (Kalinin et al. 2001). Prior to dying in the outermost cornified layers of normal skin, KCs undergo highly orchestrated molecular events during the transition from proliferative compartment in the basal layer into growth arrested early and late differentiated cells in the suprabasal layers (Watt 1989). The stratum corneum provides a vital barrier function protecting against infection and exposure to radiation and excessive water loss (Barker et al. 1991; Candi et al. 2005; Lippens et al. 2005; Boehm 2006).

New insights into the molecular mechanisms contributing to creation and maintenance of the stratum corneum are increasing our understanding of this unique form of cell death, which is shedding light into a variety of skin diseases associated with barrier malfunction (Nickoloff et al. 2002; Candi et al. 2005). Transcriptional events in KCs regulating complex processes of differentiation and host defense required to maintain constant epidermal thickness and resistance to infection in either young or aged skin, are also beginning to be better understood from skin biologists' perspective. By fractionating normal human skin into various layers (e.g. basal versus suprabasal), investigators have identified patterns of gene expression that contribute to KC

proliferation and differentiation (Watt 1989; Radoja et al. 2006). These reports indicate that the skin layers are under dynamic changing conditions, and therefore our aim in this study was to understand the molecular basis of terminal differentiation and cornification. To this end, we utilized global transcriptional analysis of KCs maintained in a three-dimensional organotypic culture system (e.g. epidermal equivalents or EE), by raising human KCs maintained on a matrix-coated membrane, from a submerged state to an air/liquid (A/L) interface.

Raising KC monolayers from a submerged state to an A/L interface generates reproducible morphological changes by which the stratified KC layers resemble normal human epidermis (Chaturvedi et al. 2006). In our previous study using monolayer cultures of KC (Perera et al. 2006a), rather than EE, global gene expression profiling revealed that early confluent KCs were somewhat similar to proliferating KCs, yet prominent differences were evident when compared to late confluent KCs; which were also distinct from replicatively senescent KCs. We report here the mRNA, miRNA and long non-coding RNA differential gene expression profiles identified by using the EE culture model.

Materials and Methods

KC Model Systems

Reconstituted human epidermis, known as submerged EE, was purchased and used following manufacturer's instructions (MatTek Corp., Ashland, MA) as previously described in our earlier report (Chaturvedi et al. 2006). To trigger KC differentiation, the EEs were raised to an air-liquid interface that reproducibly results in the creation of a granular cell layer and stratum corneum following a series of cellular and molecular events that regulate cell death such as caspases (Chaturvedi et al. 2006).

Ncode miRNA Array

We used the latest miRNA NCode version 2 commercial array (Invitrogen/Life Technologies, CA) containing 553 human and 427 mouse miRNAs and TILDA array (Life Technologies, CA) for this study. This multi-species miRNA microarray contains probes for all validated miRNAs from five species, including human, aiming to utilize species conservation. The miRNA labeling was done according to the NCode protocol AlexaFluor conjugated dendrimers. A Maui hybridization oven was used for chip processing and images were captured using the Axon B-4000 scanner.

mRNA Array U133A for Gene Expression Analysis

Total RNA from EEs at different stages (submerged, 6 hrs, 18 hrs and 24 hrs) was used for Affymetrix gene expression profiling. Each of the groups had four biologically independent replicates in each stage. Total RNA was harvested using the RNeasy kit (QIAGEN, Inc., Valencia, CA). For first and second strand cDNA synthesis, SuperScript reverse transcriptase and oligo-dT primers containing T7 RNA polymerase promoter sites were used. In vitro transcription (IVT) reactions were done according to a high yield

transcription labeling kit from ENZO (ENZO Bio Inc., New York, NY). Fifteen micrograms of cRNA were fragmented and then hybridized to an Affymetrix Human array HU-U133A chip. Samples were prepared for hybridization according to the Affymetrix Expression Analysis Technical Manual.

Non-coding RNA Array

We and our colleagues (Mattick Laboratory at the University of Queensland, Australia and Invitrogen/Life Technologies Corporation) designed a new ncRNA array platform that targets over 10,000 putative long (>200 nt) ncRNAs, including most of the known ncRNAs in mouse and human. Lack of coding potential was estimated by an algorithm that scores various characteristics of protein-coding genes, including open reading frame length, synonymous/non-synonymous base substitution rates and similarity to known proteins (Amaral et al. 2008; Dinger et al. 2008; Mercer et al. 2008). These arrays provide the first generation of tools designed to analyze the dynamic expression of a large subset of lncRNAs in human and mouse and to identify candidate genes for more detailed functional analysis. In addition to the lncRNA content, probes targeting mRNA content from RefSeq are also included, allowing discovery of coordinated expression with associated protein-coding genes.

Statistical Analysis

miRNA custom array data from a scanner consists of probe level data. A probe set contains 15 probe pairs and each probe pair consists of a perfect match and a mismatch probe. Affymetrix MAS version 5 was used to produce signal and signal detection calls. The resulting signals were log-transformed with base 2. A linear normalization with trimmed mean was employed to normalize signals across different experimental

conditions. Mean signals of four replicate chips in each group were used for fold change computation (except the 18 hrs group where three replicate chips were used because one chip failed our quality control check). For both miRNA and DNA array data, one-way analysis of variance was conducted for each miRNA or gene. Planned pair wise comparisons were also performed by comparing the mean signal in each condition to that of the 0 hrs baseline. All the statistical computations were conducted using SAS® and S-Plus®.

qRT-PCR

We used several protocols to validate the differential expression of miRNA, mRNA and lncRNA in EE samples. For miRNAs, the universal qRT-PCR starts with total RNA, including miRNAs, which polyadenylate and then reverse-transcribe with a poly(T) adapter into cDNAs for real-time PCR using a miRNA-specific forward primer and the sequence complementary to the poly(T) adapter as the reverse primer (NCode qRT-PCR protocol from Invitrogen/Life Technologies). A second protocol used was the miRNA qRT-PCR method by Applied Biosystems/Life Technologies (Chen et al. 2005). Delta delta changes were calculated and an RNU48 probe from Applied Biosystems/Life Technologies was used as a reference. Syber green-based qRT-PCR was used for the validation of mRNA and lncRNA expression.

Validation miRNA Expression by Northern Blots

Twenty ug of total RNA was concentrated from each sample (submerged, 6 hrs, 18 hrs, 24 hrs and 120 hrs), prepared in 2x TBE-Urea sample buffer, and loaded into 15% TBE-Urea acrylamide gels for electrophoresis. Gels were electroblotted to positively charged nylon membranes and cross-linked in an ultraviolent crosslinking chamber,

prehybridized with Ultrahyb-Oligo for 30 min at 42^{o}C and finally probed with 5'-biotinylated anti-miR DNA oligonucleotides and anti-U6 probes at a concentration of 10 pM, at 42^{o}C overnight. Blots were washed x2 using Low Stringency Wash Buffer (2X SSC, 0.1% SDS, Ambion) for 10 min per wash. Blots were processed using the Brightstar Detection Kit (Ambion) and developed on film (Kodak).

Results

mRNA Expression Changes in EE cultures

Human KCs differentiate into phenotypically distinct cell layers in organotypic cultures by raising cultures to an A/L interface (Harris et al. 2002; Chaturvedi et al. 2006). Time dependant morphological changes are reflected by light microscopic appearances as depicted in Fig. 1. To understand the underlying molecular mechanisms responsible for these cellular changes, we designed a series of experiments using EE samples collected from cultures and raised under progressively increasing levels of differentiation by examining EEs at various time intervals after being raised to an A/L interface. We isolated RNA at 0 hrs, 6 hrs, 18 hrs and 24 hrs, and ran Affymetrix expression arrays to examine the global gene expression profiles in the EE cultures. After 24 hrs, EE cultures differentiated into a distinct structural entity with a stratum granulosum and stratum corneum resembling human skin. Affymetrix U133A microarray analysis was done according to the statistical methods discussed in the Materials and Methods section. The results are shown in the form of principle component analysis (PCA) in Fig. 2.

PCA is a method that is commonly used in microarray research as a cluster analysis tool. It is designed to capture the variance in a dataset in terms of its principle components. In effect, one is trying to reduce the dimensionality of the data to summarize the most important (i.e. defining) parts, while simultaneously filtering out noise. The PCA shows a time dependent sample separation. The separation appears counterclockwise and may be due to differentiation related events that occur during this process. According to the PCA, almost all the replicates from the four biological independent samples are closely associated, indicating minimum variation between samples.

Those genes which showed statistically significant expression changes were further clustered by the hierarchical cluster algorithm (K-mean). Our initial analysis focused on identifying and characterizing genes that belong to cell cycle related pathways. Despite the relatively narrow window of experimental time points (0 to 24 hrs), several differentially regulated cell cycle related genes were identified at 24 hrs compared to submerged cultures including: cyclin A2 (*CCNA2*), kinesin family member 2C (*KIF2C*), MAD2 mitotic arrest deficient-like 1 (yeast) (*MAD2L1*), cyclin B1 (*CCNB1*), cyclin B2 (*CCNB2*), CDC20 cell division cycle 20 homolog (*S. cerevisiae*), cyclin-dependent kinase inhibitor 3 (*CDKN3*), *NOTCH* and *CDK6*.

To monitor the expression changes of cell death and differentiation, we extracted most of the known and previously documented apoptosis genes and looked for expression changes in the microarray dataset. Apoptosis related genes were clustered (K-mean nearest neighbor) to minimize the number of confounding variable, The clusters demonstrated several distinct expression patterns. Two such clusters are shown in

Supplemental Fig. 1. *TGM2*, *STAT2*, *FGFR2*, *ILR3RA*, *CLU*, and *IGFBP4* were all identified as down-regulated genes at 24 hrs compared to submerged cells. Clusterin or CLU expression is known to be differentially regulated in skin, and the functions of this gene include cell-cell adhesion and aggregation, inhibition of complement cytolysis, programmed cell death and apoptosis, tissue remodeling, and terminal differentiation (Seiberg and Marthinuss 1995). Apoptosis related genes that are found to be up-regulated are listed in Table 1.

Note that the large numbers of genes that are up-regulated not only contribute to the death of KCs necessary to create the stratum corneum, but also to support other cellular functions listed above. For example, NAD(P)H dehydrogenase quinone 1 is known to be differentially regulated in human epidermal KCs and also in reconstructed epidermal models (Harris et al. 2002). The receptor tyrosine kinase, KIT, has been shown to be important for the survival, proliferation, and migration of melanoblasts (Jordan and Jackson 2000). Luscher-Firzlaff, et al. (Luscher-Firzlaff et al. 1999) identified the Fork head domain transcription factor M phase phosphoprotein 2 (MPP2) as an interaction partner of E7; thus, MPP2 is a potentially important target for E7-mediated cellular transformation in skin. Additionally, Pirin (iron-binding nuclear protein) has been described as a novel regulator for KC differentiation (Burchiel et al. 2007).

The observation that KRT15 is up-regulated at 24 hrs indicates that the differentiation is an ongoing process in EEs. We examined the mRNA expression levels of all four NOTCH variants by qRT-PCR in EEs (Fig. 3) and the results indicated that both *NOTCH1* and *CDK4* are up-regulated at 24 hrs and 120 hrs compared to submerged

cultures. However, the expression of *NOTCH* 2, 3 and 4 are found to be up-regulated only at 120 hrs compared to submerged EEs.

miRNA Expression in EE Cultures

miRNAs are known to regulate the mRNA expression either by transcriptional cleavage or translational inhibition in higher eukaryotes. To examine the role of miRNAs in skin development, we analyzed their expression using two different miRNA array platforms (Affymetrix and NCode). The results indicated that there are minimal miRNA expression changes compared to mRNA expression changes in the same samples. Overall low numbers of differentially expressed miRNAs at 0 hrs, 6 hrs, 18 hrs and 24 hrs suggests that longer time points may be necessary to reveal significant changes during the differentiation process. Several statistically significant miRNAs were characterized by Northern blot analysis and the data is depicted in Fig. 4. As illustrated in Fig. 4, miRNA-203, 205 and let-7b all show an elevated expression level at 18 hrs and 24 hrs, but significantly lower expression at 120 hrs compared to submerged (0 hrs). Since we have observed an elevated expression of *NOTCH* (Fig. 3), which is a putative target gene of miR-205 (www.targetscan.org), it appears that miR-205 may correlate inversely to *NOTCH* expression levels during EE differentiation.

miRNA-205 and its Putative Target mRNA Network

To fully understand the significance of miRNA regulation in EEs and monolayer cultures, we have developed a systems level network that integrates miRNA-205 and its putative mRNA targets. We began with a list of candidate target genes of miR-205 using

computational resources including miRanda, miRbase, miRNAmap, Tarbase, PicTar, Target ScanS, and DIANA MicroTest (http://www.ncrna.org). Before experimental validation of putative targets, it is important to first computationally evaluate whether the putative target genes are biologically significant. To do this, we generated first degree interaction maps for each putative target gene. In this preliminary analysis, the putative target genes for miR-205 were imported into GeneGo software for further analysis (Fig. 5). From the most connected and representative genes in the network and pathway analyses, it seems that organogenesis, proliferation, development and cell cycle regulation themes are prevalent, along with ECM signaling, ion and/or vesicle transport. Damage response genes are also observed, and some well-known signaling cascades (MAPK, IP3) are also represented among the putative targets of miR-205.

Long non-coding RNA differential Regulation in EEs

We used a new ncRNA array platform developed by Invitrogen/Life Technologies Corporation that contains probes to target over 10,000 putative long (>200 nt) ncRNAs (lncRNAs), including most of the known ncRNAs in mouse and human. The lack of coding potential has been estimated by an algorithm that scores various characteristics of protein-coding genes, including open reading frame length, synonymous/non-synonymous base substitution rates and similarity to known proteins. These arrays provide the first generation of tools designed to analyze the dynamic expression of a large subset of lncRNAs in human and we have used them to identify EE differentiation specific lncRNAs. In addition to the lncRNA content, probes targeting mRNA content are also included, allowing discovery of coordinated expression with associated protein-

coding genes. Several differentially expressed lncRNAs were identified and their expression was confirmed by qRT-PCR (Fig. 6). Two of these lncRNAs, *AF005081* and *UC003af*, were found to be up-regulated only at 120 hrs. Contrary to this, *BC020554* was up-regulated only in the submerged EEs and down-regulated upon differentiation. A third pattern of expression changes was observed with the lncRNA *AK022798*, which revealed low expression at the submerged phase, but up-regulated for early differentiation, and then gradually declined as it progressed through differentiation.

Because only a relatively small number of lncRNAs have been characterized to date, there are as yet no established approaches by which function or regulatory targets can be predicted. Furthermore, from the lncRNAs that have been studied, it appears that their modes of action are likely to be diverse; indeed, this diversity is reflected in the four differentially expressed lncRNAs identified in this study in terms of their size, expression profile and genomic context. Nevertheless, one emerging theme that has arisen from the lncRNAs examined to date is that lncRNAs may influence the expression of nearby protein-coding genes (Mercer et al. 2009). Therefore, we examined the genomic context of the lncRNA candidates identified here to obtain some insight into their potential function.

Within the genome, *AK022798* is derived antisense to the TRAF3-interacting JNK-activating modulator gene (*TRAF3IP3*; Supplementary Fig. 2). Interestingly, this 1746 nt transcript traverses (on the antisense strand) an alternative termination site of *TRAF3IP3*, raising the possibility that this lncRNA may be involved in regulating the ratios of these isoforms in differing ontological contexts. In contrast, *BC020554* is a 3408 nt transcript that originates downstream of the transcription factor LBP-9 gene (*LBP-9*;

Supplementary Fig. 3). Such apparently 3'UTR-associated non-coding transcripts appear to be widespread in the mammalian genome (Carninci et al. 2006), and although biological function has been ascribed in some cases (Mattick 2009), a general mechanism for their action has not been described. The relatively short 416 nt *AF005081* lncRNA is transcribed from a highly conserved region in a gene desert (Supplementary Fig. 3). Although the function of such intergenic lncRNAs is very difficult to predict informatically, a large set of intergenic lncRNAs was recently functionally examined and found to be involved in diverse biological processes (Guttman et al. 2009).

In summary, our expression profiling data support a role for lncRNAs in KC differentiation and have identified a number of candidates for further functional examination. Investigation of the functions of these lncRNAs is currently underway.

Discussion

Previous studies (Radoja et al. 2006), including our work on KC differentiation (Perera et al. 2006a), demonstrate the involvement of a group of key regulatory genes (adhesion, ECM, proteolysis, metabolism, cell surface receptors and secreted signaling proteins, KC makers) in the KC differentiation pathway. Among those regulatory genes, NOTCH signaling pathway genes are known to play a major role in the regulation of differentiation programs in several vertebrate cell types including adipocytes (Garces et al. 1997; Hendrix et al. 2002; Weijzen et al. 2002; Lee et al. 2004). The connection between NOTCH and adipocyte differentiation processes is further supported by the knock-down studies of PPARgamma in adipocytes (Perera et al. 2006b). The novelty of the present study is the characterization, using both mRNA and lncRNA expression

profiling, of the three-dimensional epidermis raised to an A/L interface that mimics the natural anatomical composition of the skin.

To determine which genes are involved in epidermal stratification, Koria et al. (Koria and Andreadis 2006) performed global mRNA expression profiling of fully or partially stratified skin equivalents. Our studies focus on early time points of EE differentiation and the results show both coding and non-coding RNAs are being modulated during this early differentiation process.

In a recent study, NOTCH signaling was shown to trigger differentiation of epidermal KCs (Hendrix et al. 2002) in the EE system. With the addition of Jagged-1 peptide, it was demonstrated that all 4 NOTCH receptors could be activated in submerged EEs by raising to an A/L interface (Hendrix et al. 2002). However, the relative roles for NOTCH-1 versus NOTCH-2, 3 and 4 receptors remains to be elucidated. Here we report that NOTCH-1, 2, 3 and 4 are up-regulated at 120 hrs compared to early time points, indicating the important role of NOTCH in EE differentiation. Increased NOTCH levels at 120 hrs may be important for two reasons: 1) NOTCH may be necessary to allow the early terminal differentiation program required for cornification (ibid), and 2) NOTCH may allow cell death to proceed, which is also necessary for cornification. NOTCH signaling has been shown to influence cellular proliferation, differentiation and survival in both keratinocytes and skin. In skin, NOTCH signaling functions as a molecular switch between proliferation and differentiation during epidermal homeostasis. Therefore, these studies provide some insights in the controlling mechanism of NOTCH signaling in EE differentiation.

Our experiments also indicated that miR-205 was down-regulated at 120 hrs. Given that NOTCH-1, a computer predicted putative target of miR-205, is up-regulated at 120 hrs, a possible interaction between miR-205 and NOTCH may exist. However, further experiments are necessary to support our argument. We hypothesize that the fine-tuning of *NOTCH* receptor signaling may be achieved by an interplay of specific miRNAs that impact p53 and NOTCH-1 signaling: the two key pathways of interest to investigative skin biologists.

Finally, several lncRNAs were identified as being differentially expressed during KC differentiation by lncRNA arrays and validated by qRT-PCR. The biological functions of these lncRNAs are currently under investigation. Further studies are necessary to decipher the complex cross-talk mediated by the mRNAs, miRNAs, lncRNAs and the signaling proteins involved in epidermal KCs activated during differentiation.

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Fig. Captions

Fig. 1 Morphological Characteristics of EE Culture System

Different time points (0 hrs, 18 hrs and 24 hrs) of the cellular differentiation process indicate the morphological changes that occur in KCs within the EE cultures. Upper strata formation of the granular cell layer and stratum corneum is visible at the 6 hrs and 24 hrs time points compared to submerged cultures (0 hrs).

Fig. 2 Principle Component Analysis (PCA) of the Global mRNA Expression Levels in EE Samples

Samples are color coded according to the time points. Time points are; black - 0 hrs, green - 6 hrs, blue - 18 hrs and red - 24 hrs. PCA shows time dependent global gene expression changes in the EE samples. It also shows a counterclockwise grouping of samples, which reflects the changes in the transcriptome that, in part, is dependent on the induction of differentiation observed when EEs are raised to an A/L interface.

Fig. 3 NOTCH and CDK4 Expression in EEs

NOTCH and CDK-4 mRNA expression during EE differentiation. Expression values (fold increase) are normalized to 1 at submerged (EE 0 hrs). These genes are up-regulated at 120 hrs compared to other time points.

Fig. 4 miRNA-203, 205 and *Let*-7b Expression Validated by Northern blots

20 μg of total RNA from submerged, 6 hrs, 18 hrs, 24 hrs and 120 hrs were loaded into 15% TBE-Urea acrylamide gels for electrophoresis. Probes were designed as 5’-

biotinylated anti-miR-oligonucleotides for miRNA expression detection with anti-U6 probes as a loading control. All three miRNAs show elevated expression at 18 hrs and 24 hrs compared to 120 hrs.

Fig. 5 miRNA-205 and its Putative Target Interaction in a Systems Level Network

Network illustration of the systems level interactions of the putative target genes for miR-205. The red nodes in the map indicate direct interaction between miR-205 and their putative target genes. GeneGo software was used to build these networks.

Fig. 6 Long non-coding RNA Expression during KC Differentiation

lncRNA expression in submerged (0 hrs), 3 hrs, 6 hrs, 18 hrs, 24 hrs and 120 hrs. lncRNAs AF005081 and uc0031af are up-regulated at 120 hrs compared to other time points. Expression of AK022798 increases at 3 hrs compared to submerged but gradually decreases in subsequent time points. BC020554 is up-regulated while submerged, but expression levels decrease by other time points.

Table Caption

Table 1

Apoptosis related statistically significant (P_Values < 0.05) up-regulated genes in EE cultures at 6 hrs, 18 hrs and 24 hrs after lifting to A/L interface compared to submerged cultures (0 hrs).

Supplemental Figures

Supplemental Fig. 1

K-mean expression of differentially expressed genes in EEs.

Supplemental Fig. 2

AK022798 is derived antisense to the TRAF3-interacting JNK-activating modulator gene.

Supplemental Fig. 3

BC020554, is a 3408 nt transcript that originates downstream of the transcription factor LBP-9 gene.

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