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Analysis of calcium-inducible genes in keratinocytes using suppression subtractive hybridization and cDNA microarray

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Abstract

Terminal differentiation of skin keratinocytes is a vertically directed multistep process that is tightly controlled by the sequential expression of a variety of genes. To gain further insight into the molecular events involved in this process, we used suppression subtraction hybridization (SSH) and cDNA microarray analysis. Messenger RNAs were isolated from primary skin keratinocytes cultured in vitro after treatment with calcium and then SSH was performed. A total of 840 cDNA clones were obtained from subtracted libraries, and these cDNA clones were used to make the microarray slides. Time-course cDNA microarray analysis (1, 3, 7, and 14 days after calcium treatment) revealed the global gene expression profile during keratinocyte differentiation. Of the 840 genes tested, 290 showed a greater than twofold change in expression level at least once over four time points. The genes were clustered into six groups according to their expression pattern using self-organizing map analysis and showed the global feature of function-related regulation. The genes related to keratinocyte differentiation were markedly up-regulated by calcium treatment. In addition, a unique pattern of increase was seen in the expression of genes related to ribosomal proteins. On the other hand, transcripts involved in metabolism, DNA repair, transcription, and translation were generally down-regulated. These results demonstrate the complexity of the gene expression profile that contributes to the spatiotemporal regulation of keratinocyte differentiation.
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Keywords: Skin keratinocytes; Calcium; Suppression subtraction hybridization; cDNA microarray

Skin has a role as a protective barrier against environmental insults, such as chemicals, pathogens, and UV light [1]. Much of this protective function is dependent on the epidermis, a multilayered epithelium that is composed of various cell types, including keratinocytes and melanocytes. Keratinocytes provide the rigid stratified structure through a highly complicated and tightly regulated process of differentiation [2]. The keratinocyte progenitor cells in the basal layer proliferate and move upward; the differentiation process begins in the suprabasal layers and culminates in fully differentiated dead cells on the surface. Given that this process takes place along a pathway that leads to cell cycle arrest and terminal differentiation, specific gene sets should show precise spatiotemporally regulated expression. Many differentiation-related genes, including those encoding transglutaminases 1 and 3 (TGase 1 and 3), involucrin, cornifin, loricrin, filaggrin, and small proline-rich proteins (SPRs), have been shown to be expressed in a temporally regulated manner in keratinocyte differentiation [3–7]. Despite intensive investigations to identify and determine the regulation of numerous candidate genes, the precise gene expression profile that governs keratinocyte differentiation remains to be determined.

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Several factors have been suggested to play roles in keratinocyte differentiation in vivo and in vitro, including calcium, vitamin D, retinoic acid, and 12-*o*-tetradecanoyl-phorbol-13-acetate (TPA) [8–11]. Calcium is the best characterized of these factors as a differentiating agent for keratinocytes. It has been demonstrated that there is an extracellular calcium gradient across the epidermis, increasing toward the upper layer [12]. Furthermore, the calcium dependency is well reflected under in vitro culture conditions; keratinocytes cultured in the presence of a low concentration of calcium (0.03 mM) do not differentiate and are phenotypically similar to basal epidermal cells, but when calcium concentration is raised above 0.1 mM these cells begin to acquire the morphological and biochemical characteristics of suprabasal cells and express several differentiation-related genes [13,14].

To investigate the molecular events involved in the keratinocyte differentiation process, we chose calcium as the differentiating agent. In epidermis, it takes 14 days for keratinocytes to undergo terminal differentiation and become dead corneocytes. Although there was no direct evidence that chronologic time in vitro is exactly matched to that of in vivo conditions, we speculated that similar cellular events take place under in vitro conditions and then constructed subtractive libraries using the mRNAs isolated from keratinocytes treated with calcium for 14 days, by means of PCR-based subtraction hybridization [15]. The clones obtained were used to make microarray slides, and the temporal gene expression was investigated. Because keratinocytes show several distinctive morphological changes during the differentiation process and can be divided into several stratified layers such as basal, spinous, granular, and cornified, we chose four time points that reveal the specific situation of differentiating keratinocytes: 1 day after calcium treatment, similar to cells leaving the basal layer in the skin; 3 days for the early spinous layer; 7 days for the middle spinous layer; and 14 days for the late spinous or granular layer.

Results and discussion

Microarray hybridization for expression profiling experiments

We previously reported that several genes were identified as differentially expressed genes in the calcium-induced keratinocyte differentiation process using the technique of suppression subtraction hybridization (SSH) [15]. In this study, we decided to expand the target clones to about 3000 and analyze further their expression profile. DNA sequencing and BLAST searching showed that 840 independent cDNA clones were obtained from 3000 clones. These cDNA clones were spotted onto glass slides and used for hybridization with fluorescence-labeled probes that were prepared by reverse transcription.

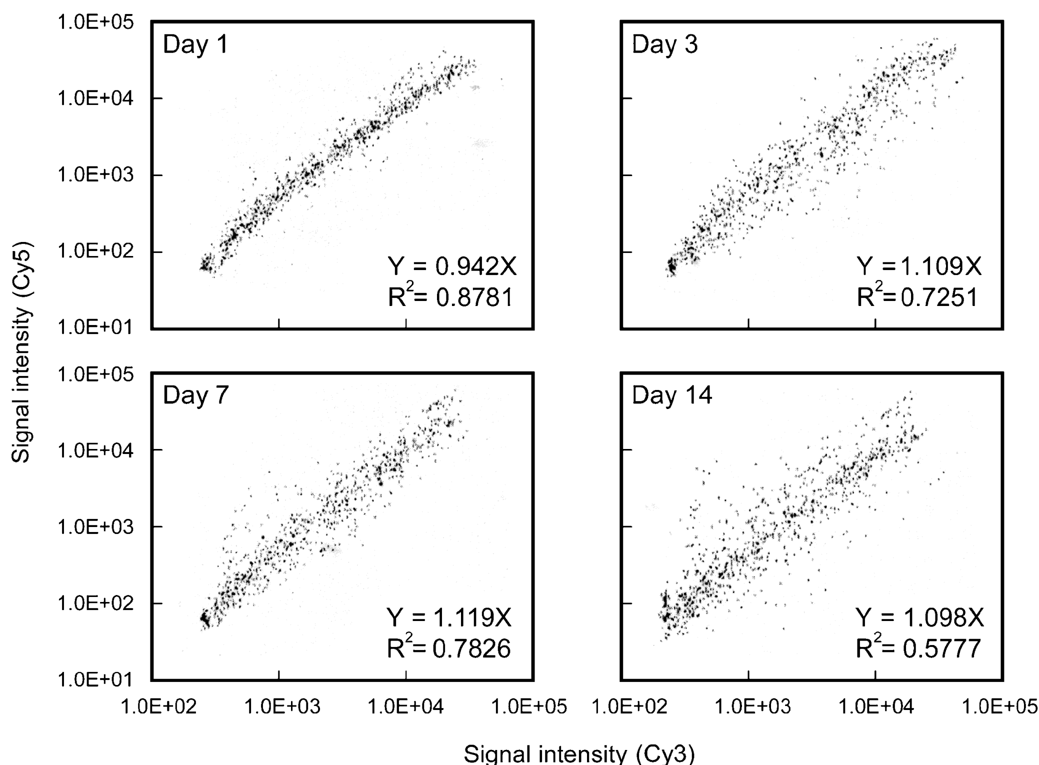

Fig. 1. Scatter-plot analysis of gene expression patterns in calcium-induced keratinocyte differentiation. Total RNAs were isolated at the indicated time points and used to make fluorescent probes. After hybridization, microarray slides were scanned and analyzed using GenePix Pro 3.0. Each gene was spotted according to the signal intensity for the control group (Cy3) vs the calcium-treated group (Cy5). Graphs are shown on the log scale.

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