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Evidence for local control of gene expression in the epidermal differentiation complex

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Abstract: The epidermal differentiation complex (EDC), located on chromosomal band 1q21, consists of at least 43 genes that are expressed during keratinocyte differentiation. Indicative of a role for chromatin structure in tissue specificity of EDC gene expression, we identified an inverse correlation between expression and DNA methylation for two EDC genes (S100A2 and S00A6) in human keratinocytes and fibroblasts. 5-azacytidine (5AC) and sodium butyrate (NaB) are two agents known to promote 'open' chromatin structure. To explore the relationship between chromatin structure and keratinocyte differentiation, we treated normal human keratinocytes (NHK) with 5AC or NaB, or with protocols known to promote their terminal differentiation. We then measured the steady-state mRNA levels for several S100 genes, small proline rich region-1, -2, and -3, loricrin, and involucrin by Northern blotting. 5AC and NaB each markedly increased expression of SPRR1/2 and involucrin in NHK. In contrast, expression of S100A2 was reduced by both agents, and by induction of keratinocyte differentiation. Moreover, while the clustered EDC genes displayed a general tendency to be expressed in epithelial cells, they displayed different patterns of cell type-specific expression. These results indicate that local, gene-specific factors play an important role in the regulation of EDC gene expression in the keratinocyte lineage and during keratinocyte terminal differentiation.

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Introduction

The epidermal differentiation complex (EDC), located on chromosomal band 1q21, consists of multiple families of clustered genes that undergo coordinate regulation during keratinocyte differentiation (1). The EDC includes members of the S100 and small proline rich region (SPRR) gene clusters and additional cornified envelope genes known to be structurally related to SPRR or S100 genes, including filaggrin, trichohyalin, and involucrin (2) (Fig. 1). Recently, the EDC has also been found to contain 18 additional genes encoding late constituents of the epidermal cornified envelope (3), the first of which was described by our group (4). In all, at least 43 genes relevant to keratinocyte differentiation are now known to be present in the EDC. Clustering of the S100 and SPRR genes is preserved in the mouse; however, the organization of the S100 gene cluster is known to differ between man and mouse (5). Two hypotheses have been ad-

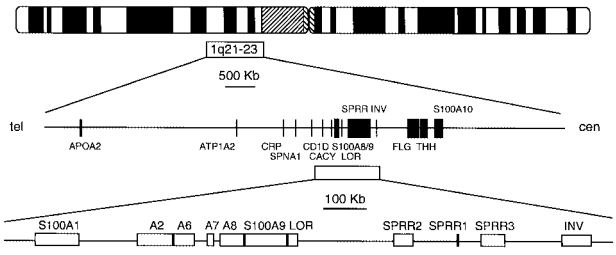

Figure 1. Map of the epidermal differentiation complex (EDC). The 1q21–23 region of chromosome 1 is expanded once to illustrate the EDC, and again to illustrate the interval spanning S100A1 and involucrin.

Abbreviations : 5AC: 5-azacytidine; EDC: epidermal differentiation complex; NaB: sodium butyrate; NHK: normal human keratinocytes; SPRR: small proline rich region.

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vanced to explain the clustered organization of the SPRR-like and S100 genes in the EDC: gene evolution via duplication and divergence, and coordinate regulation. These hypotheses are not mutually exclusive, and available evidence to date does not allow either hypothesis to be excluded.

DNA methylation at CpG sites as well as histone methylation and acetylation are epigenetic mechanisms known to regulate chromatin structure (6,7). In this study, we have determined the CpG methylation status of two EDC genes, S100A2 and S100A6, in keratinocytes and fibroblasts. Interestingly, agents known to inhibit DNA methylation (5-azacytidine, 5AC) and histone deacetylation (sodium butyrate, NaB) are also known to inhibit growth and to promote differentiation of keratinocytes (8–11). In this study, we treated keratinocytes with both of these agents and utilized two additional protocols for inducing keratinocyte differentiation *in vitro* to investigate the hypothesis that a global opening of chromatin structure might be a rate-limiting factor leading to coordinate up-regulation of clustered EDC genes. While our results do not exclude a global opening of EDC chromatin structure in keratinocytes, they do suggest that at least some of the mechanisms leading to tissue- and differentiation-specific expression of the EDC must be exerted locally on specific EDC genes themselves.

Methods

Cells and tissues

Normal human fibroblasts and normal human keratinocytes (NHK) were established from adult human skin as previously described (12). Normal human fibroblasts were grown in McCoy's 5 A medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS). Normal human keratinocytes were grown in MCDB153 medium modified (13) for high-density KC growth (Medium 154, Cascade Biologics, Portland, OR), or by coculture with irradiated mouse 3T3 fibroblasts in 3 : 1 DMEM : F12 medium supplemented with 5% FBS, insulin (5 μg/ml), EGF (10 ng/ml), hydrocortisone (0.4 μg/ml), adenine (0.18 mM), cholera toxin (0.1 nM) and antibiotics, according to the method of Rheinwald and Green (14). HaCaT cells, an immortalized but differentiation-competent keratinocyte cell line (15), were provided by Prof. Norbert Fusenig of the University of Heidelberg and grown in Dulbecco's modified Eagle's medium containing 10% FBS and antibiotics. C32 melanoma (16), K562 erythroleukemia (17), Jurkat T cell (18), HL-60 myeloid (19), and PA-1 teratocarcinoma (20) cell lines were obtained from the American Type Culture Collection (Rockville, MD) and propagated under the conditions recommended by the supplier. B lymphoblastoid cells were obtained by Epstein-Barr virus transformation of lymphocytes from the peripheral blood of a normal human volunteer, as previously described (21). Normal and psoriatic human skin was obtained from adult human volunteers by keratome biopsy, after obtaining informed consent. All procedures involving human subjects were approved by the Institutional Review Board of the University of Michigan Medical School.

Analysis of chromatin structure and gene expression

DNA methylation assays were carried out as previously described (22) by digesting genomic DNA from normal human fibroblasts, NHK, and HaCaT cells with HpaII and MspI endonucleases, which are methylation-sensitive and methylation-resistant for cleavage, respectively. To perturb chromatin structure, NHK were treated for 48 h with 1–10 μM 5AC, which inhibits CpG methylase (23), or with 1–10 mM NaB, which inhibits histone deacetylase (24). RNA isolation from cultured cells and tissue samples and Northern blotting were performed as previously described (25). cDNA clones encoding S100A2, S100A6, S100A7, S100A8, S100A9, loricrin, SPRR-1, SPRR-2, SPRR-3, and involucrin were isolated from cDNA libraries derived from human keratinocytes (Clontech, Palo Alto, CA) or human skin (4,2-6). The specificity of the various S100A probes was confirmed by hybridization to Southern blots containing each of the plasmids (data not shown). The plasmid p36B4, which encodes ribosomal phosphoprotein PO (27), served as a loading control. Purified cDNA inserts were prepared, random-primed, and used as hybridization probes for Northern blotting, as previously described (25). Quantitation of blots was performed by densitometry of autoradiograms after normalization to 36B4.

To assess the effects of NaB on nuclear size, NHK cultures were observed by phase-contrast microscopy and photographed on slide film. The slides were projected and the long axis of randomly selected cells were measured using a ruler. Size differences were assessed for significance using the Student's paired *t*-test and assuming equal variances and a two-tailed hypothesis.

Results

As a first step towards determining the role of chromatin structure in the regulation of EDC genes, we investigated the expression and methylation status of the S100A2 and S100A6 genes in HaCaT cells, NHK, and normal human fibroblasts (Fig. 2). Lack of S100A2 expression in fibroblasts was accompanied by a high degree of CpG methylation, whereas high S100A2 expression in NHK was characterized by a near-complete lack of methylation. S100A2 mRNA is expressed at substantially lower levels in HaCaT cells than in normal keratinocytes (26) (Fig. 2). Consistent with this, HaCaT cells also displayed greater CpG methylation than did NHK. The pattern of methylation also differed from that observed in normal human fibroblasts. An inverse correlation between DNA methylation and gene expression was also observed for S100A6, with HaCaT cells displaying the lowest expression and greatest methylation, and fibroblasts displaying the greatest expression and least methylation (Fig. 2).

Next, NHK were treated with 5AC and NaB, then the cells were observed for alterations in gene expression and morphology. Both agents markedly increased expression of SPRR1/2 and INV mRNAs in NHK after 48 h of treatment, which would be consistent with an opening effect upon chromatin structure. However, expression of

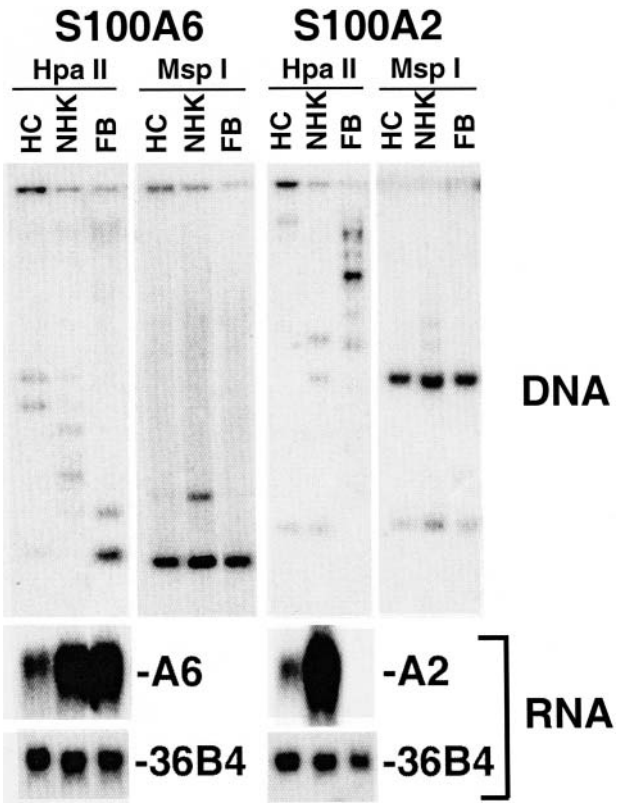

Figure 2. Expression of S100A2 and S100A6 correlates inversely with CpG methylation in keratinocytes and fibroblasts. HC, HaCaT cells. Normal human keratinocytes (NHK). Normal human fibroblasts (FB). Top panels: Southern blots of genomic DNA digested with Hpa II or Msp I as indicated at the top of the figure, hybridized against S100A6 (left) or S100A2 (right). Bottom panels: Northern blots of total RNA extracted from replicate dishes, hybridized against the probes indicated to the right of each panel. Note that within a given cell type, increasing mRNA expression (lower panels) is associated with decreasing CpG methylation producing lower molecular weight bands in the Hpa II digests (upper panels).

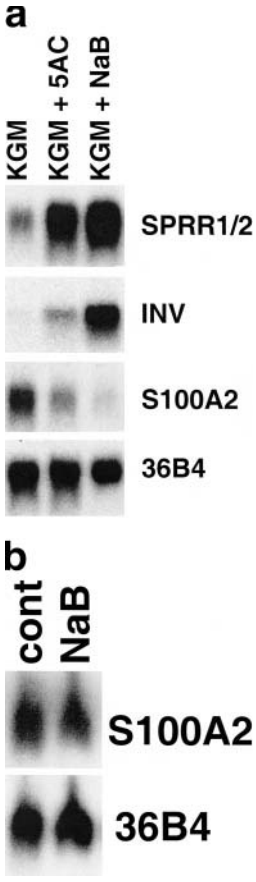

Figure 3. Differential responses of epidermal differentiation complex genes after treatment of normal human keratinocytes (NHK) with agents that promote 'open' chromatin structure. Normal human keratinocytes at 20–30% confluence *(a)* were treated for 48 h with 5AC (1 μM) or NaB (3 mM). Northern blots loaded with 10 μg total RNA are shown. Hybridization probes are indicated to the right of the blots. In the experiment shown, a mixed probe consisting of equimolar amounts of ^{32}P-labelled SPRR-1 and SPRR-2 was used to detect SPRR-1 and SPRR-2 simultaneously. Note that S100A2 is down-regulated, whereas SPRR 1/2 and involucrin are up-regulated. Effects of NaB and 5AC on S100A2 mRNA expression in HaCaT cells *(b)*. HaCaT cells at confluence were treated with NaB for 72 h. Northern blots loaded with 40 μg total RNA are shown. Hybridization probes are indicated to the right of the blots.

S100A2 mRNA was markedly reduced by both agents (Fig. 3a).

Sodium butyrate treatment of NHK caused a significant increase in nuclear size. After 48 h of treatment with 1 mM NaB, the long axis of nuclei increased by 42%, from 2.6 + 0.3 ($n = 10$) to 3.7 + 0.2 ($n = 11$) (arbitrary units) ($P = 0.0055$). No comparable increase in nuclear size was detectable after 5AC treatment.

In order to determine whether the effects of NaB might relate primarily to its effect on keratinocyte differentiation, we repeated these experiments on HaCaT cells. These cells are impaired in their ability to cease proliferating after calcium-mediated differentiation *in vitro* (15). Interestingly, NaB promoted no down-regulation of S100A2 in HaCaT cells, whereas it strongly down-regulated S100A2 in NHK. NaB also failed to produce a significant effect on nuclear size in HaCaT cells (data not shown).

We next examined whether the expression of clustered S100 genes within the EDC change in a coordinated pattern when keratinocytes are stimulated to differentiate *in vitro*. One of these methods involves the growth of NHK on fibroblast feeder layers in high-calcium, serum-containing medium (14). The other method provokes a 'psoriatic' pattern of differentiation by exposing NHK grown in low-calcium, defined medium (M154) to serum (28). These experiments revealed marked differences in the expression patterns of closely spaced

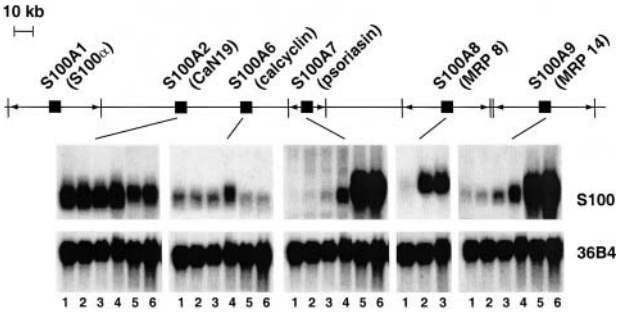

Figure 4. Differential S100 gene expression during keratinocyte differentiation. Northern blots loaded with 20 µg/lane total RNA were hybridized against the 36B4 control gene (lower panels) or against cDNA inserts derived from the genes depicted at the top of the figure (upper panels). Lane 1: Normal human keratinocytes (NHK) grown in M154. Lane 2: postconfluent Rheinwald-Green keratinocytes. Lane 3: postconfluent Rheinwald-Green keratinocytes treated for 4 h in medium containing 10 mM TPA. Lane 4: NHK grown in M154 + 0.1% fetal calf serum (FCS) for 24 h. Lane 5: NHK grown in M154 + 1% FCS for 24 h. Lane 6: NHK grown in M154 + 10% FCS for 24 h.

EDC genes. S100A2 and S100A6 underwent little or no up-regulation in response to either treatment. In fact, these transcripts were moderately down-regulated by 1% or 10% serum. As determined by densitometry, S100A2 mRNA was reduced by 40%, and S100A6 was down-regulated by 50%. In contrast, the S100A7, S100A8, and S100A9 genes were markedly up-regulated by one or both of these protocols (Fig. 4).

In order to better compare the behaviour of S100 and non-S100 genes residing within the EDC, we analyzed Northern blots from NHK and HaCaT keratinocytes, normal and psoriatic skin, melanocytes, fibroblasts, and haematopoietic cell lines derived from lymphoid, myeloid, and erythroid lineages. A teratocarcinoma cell line was also included as a representative of the germ line (Fig. 5). S100A2 and S100A6 were well expressed in undifferentiated NHK, whereas the remaining S100 genes, loricrin, SPRR1, SPRR2, and involucrin were not expressed (lane 2, Fig. 5). Multiple EDC genes were overexpressed in psoriatic lesions relative to normal skin. These included S100A2, S100A6, S100A7, S100A8, and S100A9 as well as SPRR2, SPRR1, and involucrin. Some of these changes in expression have previously been reported by the authors (29). However, S100A1 mRNA was not appreciably expressed in psoriatic skin, and loricrin mRNA was actually down-regulated in psoriatic lesions. Examining other cell types, we found that S100A1 and S100A6 were well expressed in melanoma cells, whereas the S100A2 gene, which lies between S100A1 and S100A6, was transcriptionally silent. S100A6 was the only EDC gene tested that was expressed in fibroblasts, and S100A8 and A9 were expressed in HL-60 myeloid leukaemia cells, as previously reported (30). Otherwise, expression of EDC genes appeared to be confined to keratinocytes.

Discussion

We have previously demonstrated coordinate regulation of several EDC genes in skin lesions of psoriasis (29). By use of positional cloning, we have also previously identified novel genes in the EDC that are selectively expressed only in differentiating keratinocytes (4). Our objective in this study was to determine whether long-range alterations in chromatin structure might be a rate-limiting factor leading to coordinate regulation of EDC genes in the keratinocyte lineage or during keratinocyte differentiation.

An inverse correlation was identified between gene expression and DNA methylation for the S100A2 and S100A6 genes when assessed in fibroblasts, NHK, and HaCaT cells (Fig. 2). As many studies have found a strong correlation between CpG methylation and 'closed' chromatin (31), this result indicates that chromatin structural alterations also participate in the control of genes located in the EDC. However, the S100A6 gene exists in a hypomethylated and transcriptionally active chromatin structure in fibroblasts, whereas the S100A2 gene, which resides approximately 30 kb away only, is highly methylated and transcriptionally inactive in fibroblasts. These results support gene-specific rather than global effects of chromatin structure on gene expression across the S100 gene cluster in keratinocytes vs. fibroblasts. The inverse correlation between CpG methylation and gene expression is not perfect, as comparable expression of S100A6 in NHK and fibroblasts occurs in the presence of different amounts of methylation. However, it is important to note that methylation is only one of many factors controlling the expression of a given gene. It is possible that NHK have a higher concentration of *trans*-acting factors than do FB, allowing for comparable expression of the two genes despite different levels of CpG methylation.

Sodium butyrate and 5AC treatment up-regulated the expression of SPRR-1/2 and INV genes in NHK (Fig. 3a). Given that these agents promote keratinocyte differentiation *in vitro* (8,9–11) and promote 'open' chromatin structure (32,33), these responses might be consistent with a need for chromatin opening during keratinocyte differentiation. However, both of these agents caused pronounced down-regulation of S100A2 mRNA in NHK (Fig. 3a). Two lines of evidence suggest that

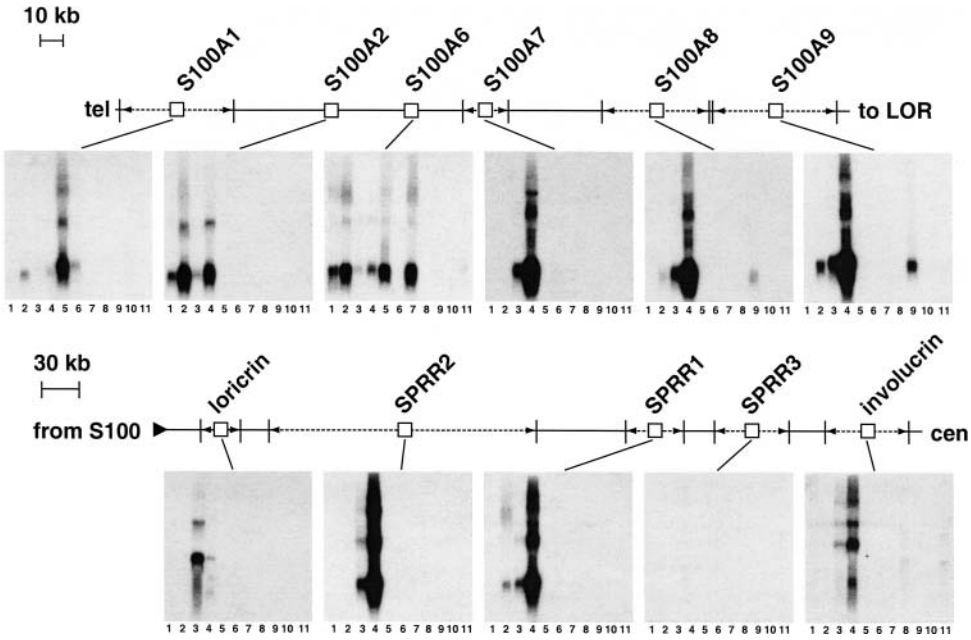

Figure 5. Differential epidermal differentiation complex (EDC) gene expression in various cell types, and in normal vs. psoriatic skin. Each lane was loaded with 40 μg total RNA. Replicate blots (three in all) were sequentially hybridized against cDNA inserts derived from the genes indicated above the autoradiograms. Key to lanes: 1, HaCaT cells; 2, NHG grown in M154; 3, normal skin; 4, psoriatic skin; 5, C32 melanoma cells; 6, K562 erythroleukemia cells; 7, normal human fibroblasts; 8, Jurkat T cells; 9, HL-60 myeloid cells; 10, PA-1 teratocarcinoma cells; and 11, Epstein-Barr virus-transformed B lymphoblasts. Each blot was also hybridized with 36B4, revealing equal loading of all lanes (data not shown).

the inhibitory effect of these agents on S100A2 mRNA is a consequence of activation of the keratinocyte differentiation program. First, NaB did not reduce S100A2 mRNA levels in HaCaT cells, which are relatively resistant to the antiproliferative effects of calcium-induced keratinocyte differentiation (15) (Fig. 3b). This finding suggests that HaCaT cells are also impaired in their capacity to undergo NaB-induced differentiation, and that the changes in S100A2 expression induced by NaB may reflect the known effects of this agent on keratinocyte differentiation, rather than a global opening of EDC chromatin structure. We and others have shown that S100A2 protein is strongly expressed in the basal cell layer *in vivo*, and markedly diminishes as keratinocytes exit the basal layer [(34); Tong Zhang and James Elder, unpublished results]. Second, S100A2 (and S100A6) were moderately (40–50%) down-regulated in NHK treated with 1 or 10% serum (Fig. 4), which is known to markedly suppress proliferation and stimulate psoriasis-like differentiation of keratinocytes (28). It is true that S100A2 continued to be expressed when keratinocytes were cultured on fibroblast feeder layers, despite the presence of serum and high calcium concentrations (Fig. 4). However, in this culture system, keratinocyte proliferation is maintained in the lowermost cell layer, while differentiation occurs in the upper layers (14).

We observed a 42% increase in nuclear diameter after two days of NaB treatment. This increase was statistically significant ($P = 0.0055$). Keratinocytes stimulated to differentiate by calcipotriol are also known to undergo an increase in nuclear size (35). Moreover, keratinocyte have been observed to increase from 107 to 193 μM^2 as a consequence of suspension-induced differentiation (36). We observed no increase in nuclear size after treatment of differentiation-resistant HaCaT cells with NaB (data not shown). Thus, the observed effect of NaB on nuclear size could reflect cell cycle arrest or some other aspect of keratinocyte differentiation, rather than a direct effect of NaB on chromatin structure by increasing histone acetylation. This increase in nuclear size could be a reflection of increased transcriptional activity, as keratinocyte differentiation *in vitro* is associated with a large increase in RNA content (37).

The interpretation that 5AC also acts on EDC genes primarily by promoting keratinocyte differentiation would also explain an apparent paradox: 5AC treatment increased expression of S100A2 mRNA in breast carcinoma cell lines (38), but decreased its expression in NHK (Fig. 3a). Methylation of CpG sites in the S100A2 gene is increased in breast carcinoma cell lines and tissues, relative to benign mammary epithelial cells (39). Up-regulation of S100A2 by 5AC reflects reversal

of this methylation in breast carcinoma cells (38). In contrast, NHK express high levels of S100A2 mRNA, and S100A2 is hypomethylated in NHK, relative to fibroblasts or to immortalized HaCaT keratinocytes (Fig. 2). Thus, it would be difficult for 5AC to further increase S100A2 mRNA levels by reversing an already-low level of methylation. Rather, we would suggest that the reduced expression of S100A2 after 5AC treatment of NHK probably reflects inhibition of keratinocyte proliferation and/or induction of keratinocyte differentiation by this agent.

We compared the expression of S100 vs. non-S100 genes of the EDC in normal vs. psoriatic skin sampled from human volunteers and in several different cell types (Fig. 5). S100 and non-S100 EDC genes did appear to be more consistently expressed in keratinocytes than in any other cell type tested. However, there were a number of exceptions to this generalization. S100A1 was strongly expressed in melanoma cells but only weakly expressed in skin and keratinocytes. S100A6 was strongly expressed in fibroblasts and melanocytes, and S100A8 and A9 were expressed in myeloid cells (Fig. 5). S100A2, which is adjacent to S100A1, was not expressed in melanoma cells as previously reported (40). We also confirmed our previous observation of widespread overexpression of EDC gene expression in psoriatic lesions compared with normal skin (29). However, two exceptions to global upregulation of EDC gene expression in psoriatic lesions were identified (S100A1 and loricrin). As previously reported (41), SPRR-3 was not appreciably expressed in keratinocytes or skin. Thus, again our results do not appear to support a 'global' mode of gene regulation across the EDC.

Taken together, our results do not support a monolithic global mode of tissue-specific gene regulation extending across the EDC. They are also inconsistent with (but do not necessarily rule out) a global opening of EDC chromatin structure across the EDC during keratinocyte terminal differentiation, as S100A2 and S100A6 were downregulated in NHK by 5AC, NaB, and induction of differentiation by serum. Overall, our findings are consistent with the emerging understanding of the gene-specific effects of coactivators such as p300 and PCAF: not only are such factors required for recruitment of the transcriptional machinery, but they are also necessary for generation of an 'open' chromatin template by virtue of their intrinsic histone acetyltransferase activities (42). Nevertheless, our results, and those of many other groups, do demonstrate a predilection for multiple EDC genes to be expressed in keratinocytes. Recently, Ragoussis and colleagues have reported that the EDC is frequently positioned external to the chromosome 1 territory in keratinocyte interphase nuclei, whereas it tends to remain within the chromosome 1 territory in cells such as lymphoblasts, in which EDC genes are not expressed (40). These findings provide evidence for higher-order organization of transcriptional activity within the keratinocyte nucleus, and suggest that the spatial proximity of EDC genes may provide a 'platform' for some level of coordinate regulation of EDC gene expression. Our results would suggest that even if such a global mechanism for opening EDC chromatin is operative in keratinocytes, gene-specific mechanisms must also be operative as well. Additional studies taking advantage of recently developed methods for the definition of chromatin structure via patterns of histone modification (7) are clearly warranted to address the regulation of EDC gene expression in greater detail.

Acknowledgements

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