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Terminal keratinocyte differentiation in vitro is associated with a stable DNA methylome

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Abstract

The epidermal compartment of the skin is regenerated constantly by proliferation of epidermal keratinocytes. Differentiation of a subset of these keratinocytes allows the epidermis to retain its barrier properties. Regulation of keratinocyte fate—whether to remain proliferative or terminally differentiate—is complex and not fully understood. The objective of our study was to assess if DNA methylation changes contribute to the regulation of keratinocyte fate. We employed genome-wide MethylationEPIC beadchip array measuring approximately 850 000 probes combined with RNA sequencing of in vitro cultured non-differentiated and terminally differentiated adult human primary keratinocytes. We did not observe a correlation between methylation status and transcriptome changes. Moreover, only two differentially methylated probes were detected, of which one was located in the *TRIM29* gene. Although *TRIM29* knock-down resulted in lower expression levels of terminal differentiation genes, these changes were minor. From these results, we conclude that—in our in vitro experimental setup—it is unlikely that changes in DNA methylation have an important regulatory role in terminal keratinocyte differentiation.

KEYWORDS
DNA methylation, epidermal differentiation, keratinocyte, methylationEPIC beadchip array, TRIM29

1 | INTRODUCTION

Epigenetics comprises the study of mechanisms that control gene expression without changing the DNA sequence, such as methylation of cytosine or post-translation modification of histones. DNA methylation is an epigenetic alteration of the mammalian genome that occurs at the 5th carbon position of cytosine (5 mC) and is mainly located at CpG dinucleotides. DNA methyltransferases (DNMTs) and ten-eleven translocation (TET) family members tightly regulate DNA methylation where DNMTs add a methyl group while TETs remove the methyl group.[1,2] Hypermethylation of promoter-associated CpG islands is associated but not restricted to gene silencing whereas

Abbreviations: Bis-PCR, bisulfite PCR; ChIP, chromatin immunoprecipitation; DMP, differentially methylated probe; DMR, differentially methylated region; DNMT, DNA methyltransferase; EDC, epidermal differentiation complex; FDR, false discovery rate; GO, gene ontology.

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promoter hypomethylation is considered to correlate with active gene transcription.[3,4] Conversely, it has been shown that increases in gene body methylation is associated with active transcription.[5] Genome-wide DNA methylation status can be profiled by a number of techniques such as whole genome bisulfite sequencing (WGBS), methylated DNA immunoprecipitation (MeDIP)[6] and Illumina beadchip arrays.[7]

DNA methylome studies on the skin are mainly conducted using in vivo human and mice skin focusing on the ageing of skin, wound healing, cancer and inflammation.[8] A key event for the formation of skin is the switch from keratinocyte proliferation to differentiation, after which the skin barrier is formed. It is important to understand how keratinocytes are regulated to switch from proliferation into differentiation.

A few studies have investigated the epigenetic regulation of keratinocyte differentiation via DNA methylation but reported seemingly contradictory results. This rendered us to perform a literature search to clarify which studies have been performed and what kind of data is available to date. This literature review is summarized in Table 1. In short, some studies claimed that DNA methylation is an important factor in epidermal differentiation,[9,10] while others conclude that there is little to no apparent connection between DNA methylation and epidermal differentiation.[11-13] Due to differences in sample origin, composition, collection, and analysis techniques, studies to date are difficult to compare. Our goal was to gain further insight in the regulatory role of DNA methylation during keratinocyte differentiation using the widely established in vitro primary human (adult) keratinocyte monolayer culture model.[14-18] We performed a genome-wide analysis of approximately 850 000 DNA methylation sites in proliferating and terminally differentiated keratinocytes in vitro using five different adult primary keratinocyte donors. Genome-wide methylation status at (but not limited to) CpG islands, enhancers, transcription factor binding sites and open chromatin was probed at single nucleotide resolution, in combination with RNA sequencing analysis to gain additional knowledge on genome-wide DNA methylation in the context of epidermal differentiation regulation.

2 | METHODS

2.1 | Monolayer cultures of human primary keratinocytes

Human primary adult keratinocytes were isolated from Fitzpatrick skin type I-III surplus skin after abdominoplasties (N = 3 donors) or breast plastic surgery (N = 2 donors). Because of privacy legislation, additional information, such as gender and age of the skin donors, was not available. Isolated keratinocytes were cultured in keratinocyte growth medium (KGM, Lonza, Walkersville, USA) and harvested for DNA at 50% cell confluence (proliferating keratinocytes), or were allowed to grow fully confluent. At this point, contact-inhibition and growth factor depletion initiates terminal differentiation. The differentiated keratinocytes were harvested 6 days after differentiation initiation, based on the elevated expression of terminal differentiation genes.[14-18] DNA was isolated using the DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer protocol and was used for MethylationEPIC beadchip array (N = 5 donors). Additionally, human primary keratinocytes were cultured identically as described above and harvested for total RNA to be used in qPCR (N = 3 donors) and RNA sequencing (N = 1 donor with technical replicates) experiments at 90% cell confluency and 2, 4 and 7 days after reaching full confluency.

2.2 | MethylationEPIC Beadchip array and analysis

DNA methylation status of over 850 000 genomic loci was assayed using the MethylationEPIC Beadchip array (Illumina, San Diego, USA) according to the manufacturer protocol. The raw measurements were converted into Beta values using R version 3.2.2 and RnBeads version 1.2.2, using default settings.[19] Using the RnBeads pipeline, unreliable low-intensity measurements and cross-reactive probes were removed ($P < .05$). 21 440 probes where one of the last 5 base pairs overlaps with a common SNP were excluded from the analysis. Only autosome targeting probes were used in the analysis. Finally, 822 386 probes were retained for differential analysis. R package "methylumi," on default settings, was used for scaling of probes to internal controls. The resulting methylation values were merged into a table and uploaded to the UCSC genome browser for visualization. Single probe methylation values were clustered using average linkage on Pearson correlation distance. Using RnBeads version 1.2.2. and the R package "limma," on default settings, donor sample pairing was taken into account for differential methylation analysis using paired Student's *t* test. Functional annotation of genes in the top1000 hypo- and hypermethylated probes was performed with DAVID.[20] Association of peaks to genes and associated GO annotation were performed with the "single nearest gene within 1 Mb" association rule.

2.3 | Total RNA isolation, real-time quantitative PCR and RNA sequencing

Total RNA was isolated with use of the FavorPrep total RNA kit (Favorgen Biotech, Taiwan) according to the manufacturer protocol. cDNA was synthesized, subsequent to DNase treatment and used for real-time quantitative PCR (RT-qPCR) with use of the MyiQ Single-colour Real-Time Detection System (Bio-Rad laboratories, Hercules, USA) for quantification with Sybr Green. All primers used in this study were obtained from Biolegio (Nijmegen, The Netherlands). See Table S1 for primer sequences. Gene expression levels were normalized to the expression of the human *RPLP0* gene and the relative expression levels of all genes of interest were measured using the $2^{-\Delta\Delta CT}$ method.[21] The RNA sequencing data in this study were generated previously by members from our group[17] and can be found at the GEO database (GSE98483).

TABLE 1 Overview of available literature regarding DNA methylation in skin or skin cells

Methodology	Species/ cells	Experimental model	Study findings related to methylation status	Ref
Radiolabeled quantification of methylation	Human foreskin keratinocytes	monolayer culture model	DNA methylation of differentiated keratinocytes is marginally less than undifferentiated keratinocytes	[13]
Modified methyl-accepting assay	Human embryonic stem cells	monolayer culture model	Reduction of 5 mC has no effect on stem cell viability or proliferation	[27]
HTF[a] island size analysis	Human fibroblasts and keratinocytes	monolayer culture model	CpG methylation and EDC gene expression is inversely correlated	[11]
Bisulfite sequencing	Human epidermoid carcinoma cells	monolayer culture model	S100A6 gene is methylated in non-expressing cells	[33]
MSP[b]	HaCaT keratinocytes	monolayer culture model	CB1 receptor-dependent DNA methylation leads to keratinocyte differentiation	[34]
MeDIP[c]-sequencing	Human foreskin keratinocytes	monolayer culture model	DNA methylation is important for self-renewing somatic tissue	[10]
MSP[b]	Human full skin biopsies	in vivo skin tissue	Gene-specific hypermethylation can cause abnormal cell differentiation	[35]
DNA methylation microarray	Human foreskin keratinocytes	epidermal fragmentation model	Differentially methylated genes play no role in keratinocyte differentiation	[31]
Illumina HumanMethylation27 beadchip	Human full skin biopsies	in vivo skin tissue	CpG methylation separates psoriasis skin from normal skin	[36]
Bisulfite sequencing	Mouse skin biopsies and blood cells	in vivo skin tissue, blood cells	Stem cell differentiation is associated with small changes in DNA methylation	[9]
Modified methyl-accepting assay	HaCaT keratinocytes	monolayer culture model	Phytocannabinoids can control keratinocyte proliferation and differentiation	[37]
MeDIP[c]- and bisulfite sequencing	Human full skin biopsies	in vivo skin tissue	DNA methylation patterns associate with psoriasis	[38]
ChIP-bisulfite sequencing	Human embryonic stem cells	monolayer culture model	Alterations in DNA methylation are lineage-specific	[39]
MethylC bisulfite sequencing	Human embryonic stem cells	monolayer culture model	Epigenetic mechanisms regulate early and late stages of stem cell differentiation	[40]
MeDIP[c]-sequencing	Human CD4 + T cells	in vivo whole blood	DNA methylation status in CD4 + T cells might associate with psoriasis pathogenesis	[41]
Illumina HumanMethylation27 beadchip	Human full skin biopsies	in vivo skin tissue	DNA methylation is associated with atopic dermatitis	[42]
MeDIP[c] and hMeDIP[c]-sequencing	Human foreskin keratinocytes	monolayer culture model	Dnmt3a and Dnmt3b bind and regulate active enhancers	[30]
Bisulfite sequencing	Human adult keratinocytes	monolayer culture model	Altered EDC genes expression is not accompanied by changes in DNA methylation	[12]
MethQTL and SNP analysis[d]	Human full skin biopsies	in vivo skin tissue	Association data suggest a DNA methylation-mediated genetic risk for psoriasis	[43]
MethQTL and SNP analysis[d]	Human full skin biopsies	in vivo skin tissue	Association data suggest nine DNA methylation loci for psoriasis	[44]
Illumina HumanMethylation 450k beadchip	Epidermis isolated from full skin biopsies	in vivo skin tissue	Reduced DNA methylation defines skin ageing	[45]
ELISA against 5-mC and 5-hmC	Human full skin biopsies	in vivo skin tissue	Sun exposure does not alter DNA methylation	[46]
Illumina HumanMethylation 450k beadchip	Human full skin biopsies	in vivo skin tissue	DNA methylation is associated with known SNPs for nevus count	[47]

(Continues)

TABLE 1 (Continued)

Methodology	Species/ cells	Experimental model	Study findings related to methylation status	Ref
MeDIP[c] and hMeDIP[c]-sequencing	Mouse skin cancer cells	monolayer culture model	Dnmt3a and Dnmt3b loss promotes squamous transformation via PPAR-gamma	[48]
Targeted Bis-PCR and Illumina 450k array	Human, dog, mouse (and more)	publicly available data	DNA methylation of certain CpGs is correlated to age	[49]
MethQTL and SNP analysis	Human full skin biopsies	in vivo skin tissue	No significant alteration in DNA methylation age between lesional and non-lesional psoriasis skin	[50]
MSP[b]	Human cancer cell lines	publicly available data	Mutations in tumor cell lines associate to DNA methylation instability	[51]
Targeted Bis-PCR	Human full skin biopsies	in vivo skin tissue	CG islands can function as enhancer for gene expression	[52]
Reduced representation bisulfite sequencing	Human full skin biopsies	in vivo skin tissue	Differential methylation in psoriasis candidate genes	[53]
RNA bisulfite sequencing	Human foreskin keratinocytes	monolayer culture model	Loss of DNA methylation coordinates epidermal differentiation	[54]
Illumina HumanMethylation 450k beadchip	Human cutaneous melanoma biopsies	publicly available data	Established a DNA methylation biomarker for cutaneous melanoma	[55]
Bisulfite sequencing	Human full skin biopsies	in vivo skin tissue	DNA methylation level of WNT1 is decreased in SLs	[56]
Illumina MethylationEPIC beadchip	Human full skin biopsies	in vivo skin tissue	HPV-induced warts show differential DNA methylation compared to normal skin	[57]
Illumina HumanMethylation 450k beadchip	Human full skin biopsies	in vivo skin tissue	Genes involved in AD-related processes are differentially methylated	[58]

[a]HpaII tiny fragments,
[b]methylation-specific PCR,
[c](hydroxy)methylated DNA immunoprecipitation,
[d]methylation quantitative trait loci.

2.4 | siRNA knock-down

Human primary keratinocytes were grown in KGM to 10%-15% confluency before 500 nM of Accell human SMARTpool against TRIM29 (#E-012409-00; Dharmacon, Lafayette, CO, USA) or Accell Non-targeting Control Pool (#D-001910-10; Dharmacon) was added for 48 hours. The SMARTpool siRNAs consist of three different siRNAs each. Culture medium was subsequently refreshed and supplemented with TRIM29-targeting or non-targeting siRNA SMARTpool for another 48 hours. Next, the keratinocytes (at ~90% confluency) were refreshed with KGM lacking growth factors supplemented again with the SMARTpool of TRIM29-targeting or non-targeting siRNA. Thereafter, the keratinocytes were allowed to differentiate for 7 days and were harvested for transcriptional analysis and Western blotting. TRIM29 knock-down was validated through RT-qPCR (Figure 2E, Figure S2A) and Western blotting (Figure 2F).

2.5 | Western blotting

Keratinocytes were lysed using RIPA lysis buffer and sonicated using the Bioruptor Pico system. The lysates were centrifuged at maximum speed (15 450 *g*), and the supernatant, containing the protein extract, was collected for Western blotting. The proteins were separated by SDS-PAGE and transferred to PVDF membranes using the NuPAGE system (Life Technologies, Carlsbad, USA). SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, USA) was used for chemiluminescent detection by the Bio-Rad Universal Hood Gel Imager (Bio-Rad laboratories). Antibodies used in this study: anti-actin (Sigma-Aldrich, Saint Louis, USA, clone AC-15, 1:100 000) and anti-TRIM29 (Thermo Fisher Scientific, #PA5-30488, 1:3000).

2.6 | Availability of data and materials

The methylationEPIC array data are uploaded to NCBI GEO and are available under accession number GSE144669. RNA sequencing data are available via the NCBI GEO database (accession number GSE98483).

3 | RESULTS

The DNA methylome of five human primary keratinocyte donors was analysed to study the involvement of DNA methylation in the switch

from keratinocyte proliferation to differentiation. Additionally, we investigated the correlation between transcriptome changes and DNA methylation upon keratinocyte differentiation.

3.1 | In vitro differentiation of keratinocytes is not accompanied by DNA methylome changes

Illumina's MethylationEPIC beadchip array comprises roughly 850 000 probes distributed over the genome and representing CpG islands, enhancers, transcription factor binding sites and open chromatin, providing insight into the genome-wide methylation status of proliferating and terminally differentiated keratinocytes. After probe filtering, 822 386 sites were used for methylation analysis. Clustering of methylation values revealed that the magnitude of change in methylation in terminally differentiated primary keratinocytes smaller than the inter-individual differences (Figure S1). Analysis of the genome-wide methylation status of proliferating and differentiating keratinocytes revealed no statistically significant differentially methylated regions (DMRs; not shown) and only two significant differentially methylated probes (DMPs).

To investigate whether the minor methylation changes had functional consequences, we performed differential gene expression analysis by RNA sequencing. In human, the chromosome 1q21 region, referred to as the epidermal differentiation complex (EDC), contains many genes that are important for keratinocyte differentiation, such as *filaggrin* (*FLG*), *involucrin* (*IVL*) and *small proline-rich proteins* (*SPRRs*). Indeed, these genes were found strongly upregulated during keratinocyte differentiation, indicating the terminal differentiated status of the keratinocytes in our in vitro culture system. Nevertheless, their DNA methylation status did not change between proliferating (Day0) and terminally differentiated (Day6) keratinocytes as illustrated through the UCSC genome browser (Figure 1).

Next, we zoomed in on the two differentially methylated probes and surrounding regions or genes they potentially regulate (Figure 2). Although the two DMPs showed a significant change in DNA methylation (false discovery rate adjusted *P-value* < .05) a corresponding statistically significant change in RNA expression of nearby genes before and after differentiation was not detected in our RNA sequencing data set. One of the DMPs, depicted in Figure 2B, is situated in a non-annotated region with no gene expression, and therefore, the change in DNA methylation at this CpG is unlikely to have a regulatory function. The other DMP is located inside the TRIpartite Motif Protein (TRIM)29 locus and showed transient gene expression two days after the start of keratinocyte differentiation in our RNA sequencing data set (Figure 2A shows the area surrounding the DMP, whereas Figure 2C shows gene expression dynamics across the whole *TRIM29* locus). RT-qPCR data validated the increased *TRIM29* expression at day2 and showed that TRIM29 gradually increases during further keratinocyte differentiation (Figure 2D), in line with a possible role for TRIM29 in keratinocyte differentiation as previously reported.[22]

To further address these observations and investigate the potential role of TRIM29 in the differentiation programme of keratinocytes towards becoming corneocytes, we performed *TRIM29* knock-down experiments prior and during the induction of keratinocyte differentiation for three donors. *TRIM29* knock-down throughout the experiment (Figure 2E and Figure S2A) resulted in loss of protein expression (Figure 2F) and led to lowered expression levels of major epidermal differentiation genes, for example *FLG*, *LOR*, *IVL*, *HRNR*, *TGM1*, *TGM3* and *KRT10* (Figure 2G and split per donor to illustrate donor variability in Figure S2B) at day 7 of differentiation. Although the individual genes were not significantly downregulated (except for *TGM3*), the trend towards lowered gene expression upon *TRIM29* knock-down is clear and significant for this group of genes (*P-value* .0002, Figure 2G). Although the difference in gene expression (Figure 2G, <2 fold) is minor, the observed demethylation and increased expression levels of *TRIM29* may have a functional consequence for the keratinocyte differentiation process to increase the efficiency of the differentiation process.

Although our data contained only two significant DMPs, we performed a metabolic pathway analysis based on gene ontology (GO) of the top1000 most differentially methylated gene body probe regions, separately for hyper- and hypomethylation, to rule out any global effects we overlooked because of non-significant DMPs. This strategy allows for the identification of general pathways that may be regulated by a combination of DMRs and/or DMPs. The hypermethylated probe regions revealed 26 enriched GO terms of which 4 were still statistically significant after Benjamini multiple testing correction (Figure S3, upper panel). Processes include the "G-protein coupled receptor signalling pathway," "detection of chemical stimuli" and "defense response to bacterium." For the top1000 hypomethylated probes, this analysis delivered 49 significant GO terms of which 19 were still significant after multiple testing correction (Figure S3, lower panel) and included for example "nucleosome assembly," "telomere organization" and "chromatin silencing at rDNA."

3.2 | Gene expression changes during keratinocyte differentiation do not correlate with DNA methylation status

To assess whether the aforementioned methylation-related GO terms are functionally relevant for transcriptional regulation, we examined the correlation between change in methylation and change in gene expression during the process of keratinocyte differentiation on a genome-wide scale. For these experiments, we used a RNA sequencing data set that was published before by our group and for which we showed that it represents both proliferating and fully differentiated keratinocyte samples.[17] We first correlated the DNA methylation changes of the single CpG sites with more than 10% difference in DNA methylation (380 probes) to the gene expression changes of the single nearest gene during differentiation (Figure S4A). A correlation was not observed (correlation coefficient of −0.087). However, this might be due to the fact

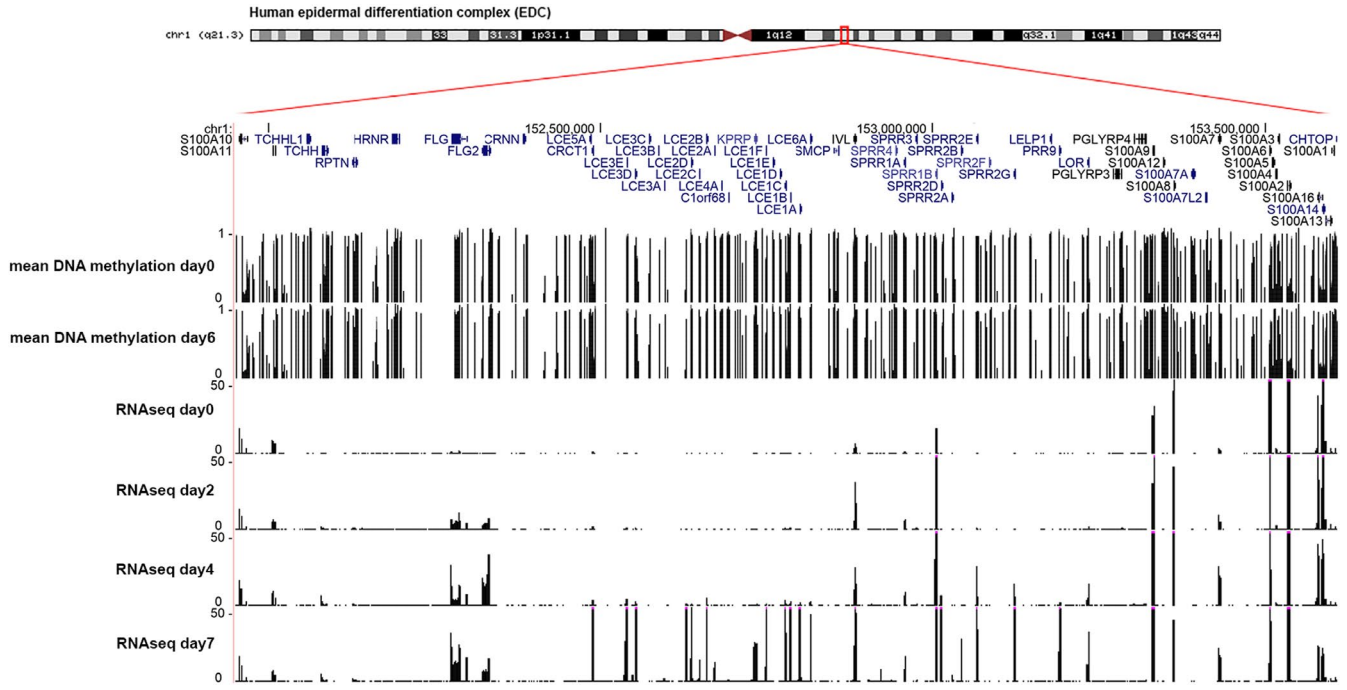

FIGURE 1 Keratinocyte differentiation is associated with a stable DNA methylome. The epidermal differentiation complex (EDC) on chromosome 1q21 contains many differentiation genes such as *FLG*, *LCEs*, *SPRRs*, *S100s*). The DNA methylation status between day0 and day6 is highly similar as shown by the genome browser tracks (mean DNA methylation day0 and mean DNA methylation day6), although gene expression is induced as depicted by the genome browser tracks of RNA sequencing over four subsequent time points (RNA-seq day0 – RNA-seq day7)

that there are several CpG sites present in the same gene region that might have converse effects on gene expression.[5] Therefore, we correlated the DNA methylation changes of the top1000 dynamically methylated gene promoter regions (Figure S4B) and the top1000 gene body regions (Figure S4C) to the gene expression changes during differentiation. Again, there was no correlation in both regions, shown by the respective correlation coefficients of −0.057 and 0.003.

4 | DISCUSSION

Here, we have focused on the regulatory role of DNA methylation in keratinocyte differentiation. The differentiation programme of keratinocytes is represented by a switch in transcriptional programme including a switch in differentiation-linked genes. The conventional monolayer culture of keratinocytes used in this study is a highly standardized, reproducible and widely accepted experimental model for over four decades to study epidermal proliferation and differentiation processes in a controlled environment.[15] As illustrated in our experiments for the EDC gene region (Figure 1), in Figure S2B (siCtrl condition), and in various publications,[16-18] upon reaching full confluence, cultured keratinocytes exit the cell cycle and switch to a terminal differentiation programme in becoming corneocytes. The keratinocytes gradually start expressing several differentiation markers such as *filaggrin* (*FLG*), *involucrin* (*IVL*), *loricrin* (*LOR*) and *hornerin* (*HRNR*) while the expression of proliferation markers such as *keratin 5* (*KRT5*), *keratin 14* (*KRT14*) and *marker of proliferation Ki-67* (*MKI67*) is decreasing.

In the current study, using this monolayer culture model, we detected a stable DNA methylome when comparing proliferating and terminally differentiated human primary keratinocytes, as indicated by a lack of differentially methylated regions. Out of 822 386 probes, we found two differentially methylated probes, one of which is situated in the *TRIM29* locus. The functional studies we performed indicate a potential role for TRIM29 in the regulation of keratinocyte differentiation, as shown by marker gene expression. Although there is some debate on the role of TRIM29 in epithelial-mesenchymal transition in cancers,[23,24] literature on TRIM29 in keratinocytes is limited. A previous study reported on the role of TRIM29 in keratinocyte differentiation after UVB exposure,[22] which is in line with our findings on a possible role for TRIM29 in a keratinocyte differentiation model without exposure to UVB. Loss of TRIM29 expression was recently reported to alter keratin expression which eventually promotes cell invasion in squamous cell carcinoma.[25] TRIM29 deficiency in HaCaT keratinocytes resulted in a loss of stratification of epithelial tissue and migration and invasion of TRIM29 negative keratinocytes that not differentiate but remain proliferative. These data and our results thus direct towards a role for TRIM29 in regulating the transition towards differentiation in keratinocytes. However, considering the magnitude of differences in expression levels of TRIM29 in our study and the subtle effect of TRIM29 knock-down in our functional assays, we suggest that TRIM29 is more likely to play a role in the fine-tuning of the differentiation process in conjunction

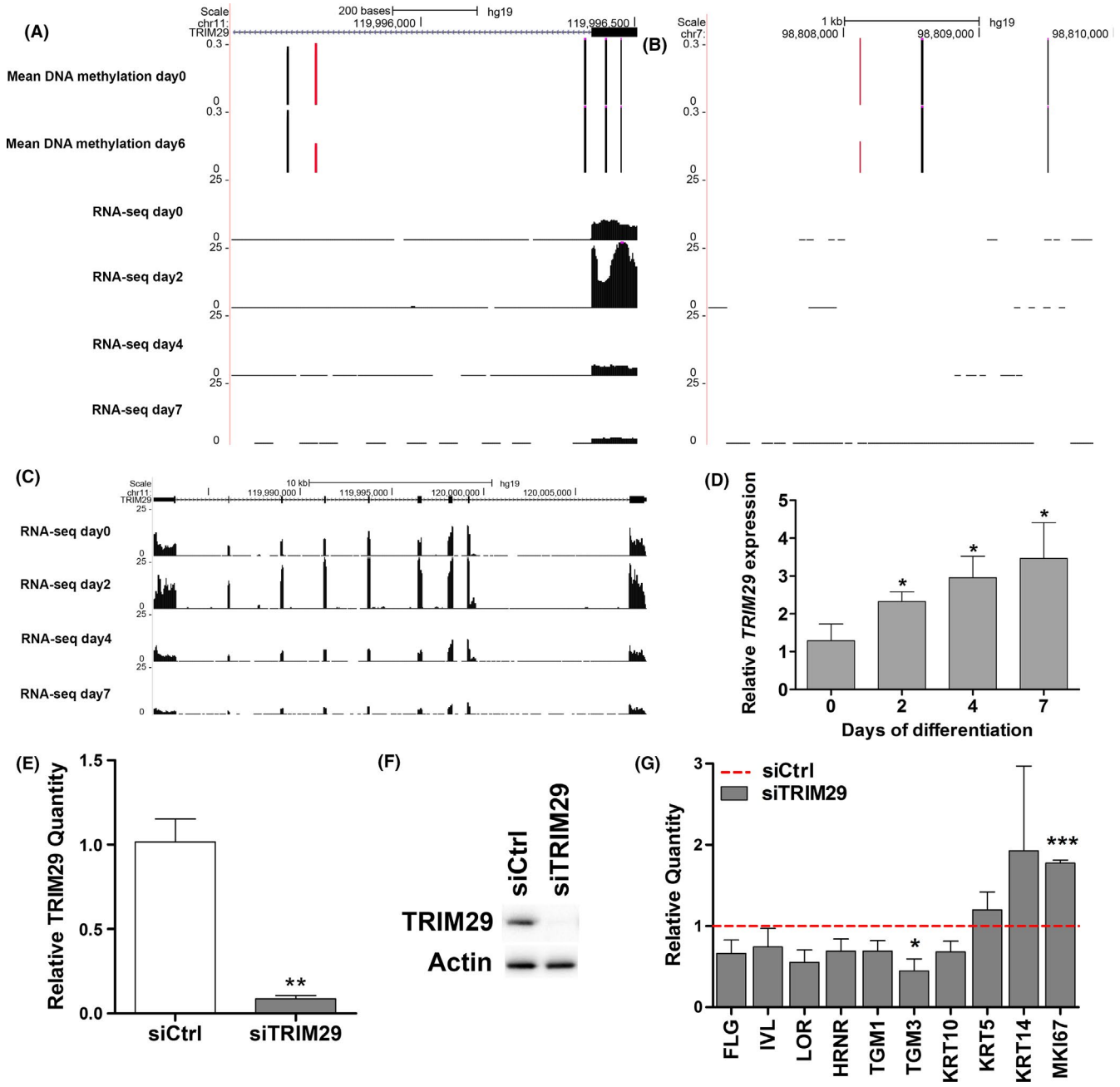

FIGURE 2 Differentially methylated probes upon differentiation of primary keratinocytes and siRNA targeted knock-down of TRIM29. A, B, C, UCSC genome browser screenshots showing two significantly differentially methylated probes (in red) in the MethylationEPIC beadchip array. Both examples show hypomethylation on day6 in comparison with day0. The RNA sequencing genome browser tracks at the bottom of panel A and C show *TRIM29* expression which in comparison with day0 is increased at day2, but declines at day4 and day7. The unannotated gene in panel B is not expressed. D, Transcriptional expression induction of *TRIM29* as analysed by RT-qPCR. E, SMARTpool siRNA against TRIM29 mediated the knock-down of *TRIM29* expression at day7 of keratinocyte differentiation, shown as mean gene expression for 3 primary keratinocyte donors. F, Western blot shows TRIM29 protein depletion upon siRNA treatment, in contrast to non-targeting siRNA (siCtrl). G, Subsequent to TRIM29 knock-down, gene expression of several differentiation genes (*FLG*, *IVL*, *LOR*, *HRNR*, *TGM1*, *TGM3* and *KRT10*) was assessed after 7 d of differentiation. Although single genes (except for *TGM3*) are not significantly lowered, the set of genes is as follows: *P-value* .0002. Expression of genes associated with proliferating (basal) keratinocytes, such as *KRT5*, *KRT14* and *MKI67*, was elevated upon *TRIM29* knock-down. **P-value* < .05, ***P-value* < .01. ****P-value* < .001. N = 5 primary keratinocyte donors (A,B,C,D) and N = 3 primary keratinocyte donors (E,F,G)

with other regulatory proteins instead of being a master regulator of epidermal differentiation by itself.

GO analysis on the top1000 ranking differentially methylated regions of our data set shows alteration of biological pathways that are relevant in the context of cellular differentiation, cell cycle processes and protection against bacteria. For example, we observed hypermethylation of genes that are involved in the detection of chemical stimuli, keratinization, several lipid metabolic processes and defense

response against bacteria. Hypermethylation of promoters in general correlates to transcriptional repression and downregulation of the corresponding biological pathways, which seemingly contradicts the differential gene expression patterns that are occurring upon keratinocyte differentiation. As the DNA methylation status of proliferating and differentiated keratinocytes seems highly similar, the biological significance of the GO term analysis remains unclear.

To put our results into perspective, we performed a thorough literature search and found that several studies have investigated the epigenetic regulation of keratinocyte differentiation via DNA methylation, but reported seemingly contradictory results. In general, DNA methylation is thought to be important in mammalian cells and during cell development.[26] In mice, for example, *Dnmt1* and *Dnmt3b* knockouts were lethal either before or just after birth.[27-29] Murine epidermal stem cells have also been analysed, and small changes of DNA methylation were shown upon differentiation.[9] In (adult) human epidermal cells, the importance of DNA methylation has been studied as well. For example, human DNTM3A induces enhancer DNA hydroxymethylation while DNMT3B promotes enhancer DNA methylation, together regulating epidermal stem cell homeostasis.[30] In addition, upon calcium-induced differentiation of neonatal foreskin keratinocytes, several differentiation gene promoter regions showed decreased DNA methylation.[10] In contrast, between human proliferating keratinocytes and differentiating keratinocytes, 18 genes were differentially methylated but none of these genes play a role in keratinocyte differentiation.[31] Furthermore, no correlation was found between altered gene expression patterns and major changes in promoter DNA methylation of *S100* and other EDC genes.[11,12] These studies are largely in line with our genome-wide DNA methylome study in which we furthermore found that inter-donor differences in DNA methylation seem larger than differences upon keratinocyte differentiation (Figure S1). In addition, the total DNA methylation percentage of undifferentiated keratinocytes was shown to be only marginally higher than the methylation percentage of differentiated keratinocytes (3% vs 1%).[13]

The discrepancies between existing studies make them challenging to interpret and put in context, considering various experimental designs and technologies used to study DNA methylation, as well as the different keratinocyte cell sources. For example, keratinocytes of neonatal foreskin origin show enhanced stem cell-like properties,[32] which could render them to behave different when compared to the adult keratinocytes we used in our study. In neonatal keratinocytes, differentially methylated gene promoter regions might be required for the switch from cell proliferation to differentiation, while adult primary keratinocytes have already been switched to a more differentiated and mature phenotype during development and ageing in vivo. Although in vitro cultures system allows for a tightly controlled environment, it also comes with limitations. For example, the in vitro monolayer keratinocyte culture model takes about a week before the keratinocytes are fully differentiated based on their transcriptome (eg the EDC genes in Figure 1), while in vivo, epidermal keratinocyte renewal takes about 30 days and regulatory processes may thus be different.

Regarding the technologies used, we applied the Illumina Infinium MethylationEPIC array to analyse 850 000 methylation probes that are located all over the genome covering enhancers, transcription factor binding sites and open chromatin on single nucleotide resolution. Others have, for example, used the MeDIP technique which makes use of an antibody to precipitate foci of highly methylated and nearby localized cytosines which therefore highly preferentially selects for promoter regions.[6] Sensitivity and specificity of these techniques vary which hinders a methodical comparison that could explain the diverse study outcomes.

Taken together, although the exact role of DNA methylation in human epidermal renewal and terminal differentiation remains elusive, we conclude that in the standardized in vitro monolayer keratinocyte culture model, human adult primary keratinocytes can terminally differentiate without the need for alterations to their DNA methylation status.

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CONFLICT OF INTEREST

The authors declare no conflicts of competing financial interests.

AUTHOR CONTRIBUTIONS

JSm, JQ, MO, PZ, JSc and EB conceptualized. RD and JQ lead data curation. JSm, RD and JQ equally did formal analysis. JSc, PZ and EB funding acquisition. JSm, RD, JQ and MO involved in investigation. JSm, RD, JQ, AB, HZ, HM and EB lead the methodology. EB lead administrated the project. Software: RD (equal) and JQ (equal). EB (lead), HZ and HM involved in supervision. JSm involved in validation. JSm, RD and JQ involved in visualization. JSm wrote original draft: JSm (lead), RD, JQ, PZ and EB wrote, reviewed and edited. All authors have critically read and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Figure S1. DNA methylation differences are independent of the differentiation status. Clustering of methylation values shows that inter-patient differences were larger than the change in DNA methylation after differentiation of primary keratinocytes as illustrated by clustering of day0 and day6 for each independent donor. N = 5 primary keratinocyte donors

Figure S2. Keratinocyte differentiation, TRIM29 knockdown, and donor variation. Keratinocytes in monolayer cultures were allowed to differentiate under non-targeting SMARTpool siRNA (siCtrl) or *TRIM29* targeting SMARTpool siRNA (siTRIM29) conditions. (A) Shows *TRIM29* knockdown efficiency per keratinocyte donor at start (day0) and after (day7) of keratinocyte differentiation. (**B**) Illustrates gene expression at start (day0) and after (day7) keratinocyte differentiation to validate the monolayer keratinocyte differentiation model and to show the effect of TRIM29 knockdown upon differentiation. Differentiation genes *FLG*, *IVL*, *LOR*, and *HRNR* are induced, while genes related to basal (proliferating) keratinocytes: *KRT5*, *KRT14*, and *MKI67* are reduced upon keratinocyte differentiation. Data are shown per keratinocyte donor to show inter-donor variability. N = 3 primary keratinocyte donors

Figure S3. Gene Ontology analysis. Metabolic pathway analysis based on gene ontology (GO) of the top1000 differentially hyper- and hypomethylated gene probe regions shows GO terms, ranked by their Log10 *P-values*. Benjamini multiple testing correction was used to calculate false discovery rate (FDR). Dark grey bars depict statistically significant GO terms after FDR correction

Figure S4. DNA methylation does not correlate to transcriptome alterations in vitro. (A) Correlation plot showing no correlation of single CpG site DNA methylation to changes in the transcriptome. (B) The top1000 dynamically methylated gene promoter regions and (C) the top1000 dynamically methylated gene body regions show no correlation of DNA methylation to changes in the transcriptome

Table S1. Primer sequences

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