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Markers for Ca^{++}-induced terminal differentiation of keratinocytes in vitro under defined conditions

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Abstract

Differentiation of normal human keratinocytes (NHK) grown in vitro as a monolayer to confluency can be triggered with an acute increase in concentration of extracellular Ca^{++}. Over several days, induced by Ca^{++}, the cells form pseudostratified sheets that somewhat resemble the basic organization of the intact skin. This experimental system is widely used in studies of keratinocyte biology and skin pathology. However, expression pattern of the genes considered as markers for cells in specific layers of epidermis in vivo does not always match the specific pattern observed in vitro and might lead to misinterpretation of data. Here, we demonstrate that among 18 markers of terminally differentiated keratinocytes of stratum granulosum (SG) and stratum corneum (SC) in vivo, only four (*CDSN*, *KPRP*, *LCE1C* and *SPRR4*) have reproduced their expression pattern in vitro. Our data suggest that findings based on two-dimensional (2D) Ca^{++}-induced terminal differentiation of NHK in vitro should be subjected to additional scrutiny before conclusions could be made and, if possible, verified in other experimental system that might more faithfully represent the in vivo microenvironment.

KEYWORDS

Ca^{++}-induced terminal differentiation in vitro, keratinocytes, quantitative PCR, reference genes

1 | BACKGROUND

Stratification and terminal differentiation of normal human keratinocytes (NHK) grown in vitro has been demonstrated more than 40 years ago.[1,2] Several factors have been suggested to play roles in keratinocyte differentiation in vivo and in vitro, including vitamin A,[3] vitamin D,[4] retinoic acid[5] and 12-o-tetradecanoylphorbol-13-acetate (TPA).[6] Among multiple factors investigated as a differentiating agent for keratinocytes, Ca^{++} ended up being the best characterized.[7] Indeed, it has been demonstrated in vivo that there is an extracellular Ca^{++} gradient across the epidermis, increasing towards the upper layer.[8] Optimization of the culture conditions and defining >1.2 mmol/L Ca^{++} as a factor driving the differentiation in a serum-free media followed.[9] The first three-dimensional (3D) skin equivalents have been reported at about the same time.[10] They consisted of a dermal equivalent, which is built from fibroblasts grown in a collagen matrix and epidermal equivalent that was made from spontaneously stratified

keratinocytes plated on the surface of the dermal equivalent. Even though the 3D system recapitulated more closely tissue in vivo, traditional two-dimensional (2D) cell culture platforms have been still utilized widely in studies of keratinocyte biology and molecular events underlying various skin diseases, probably because the experimental setting was relatively simple, and it could provide indicative information.

In the epidermis, it takes 14 days for keratinocytes from stratum basale (SB) to terminally differentiate into corneocytes of the stratum corneum (SC). Although, induced by Ca^{++}, similar events take place in vitro, there is no clear evidence that the timing in vitro is exactly matched to that of in vivo conditions. Immunodetection of SG/SC markers often serves as proof that the keratinocytes are terminally differentiated. However, it is rarely taken into account

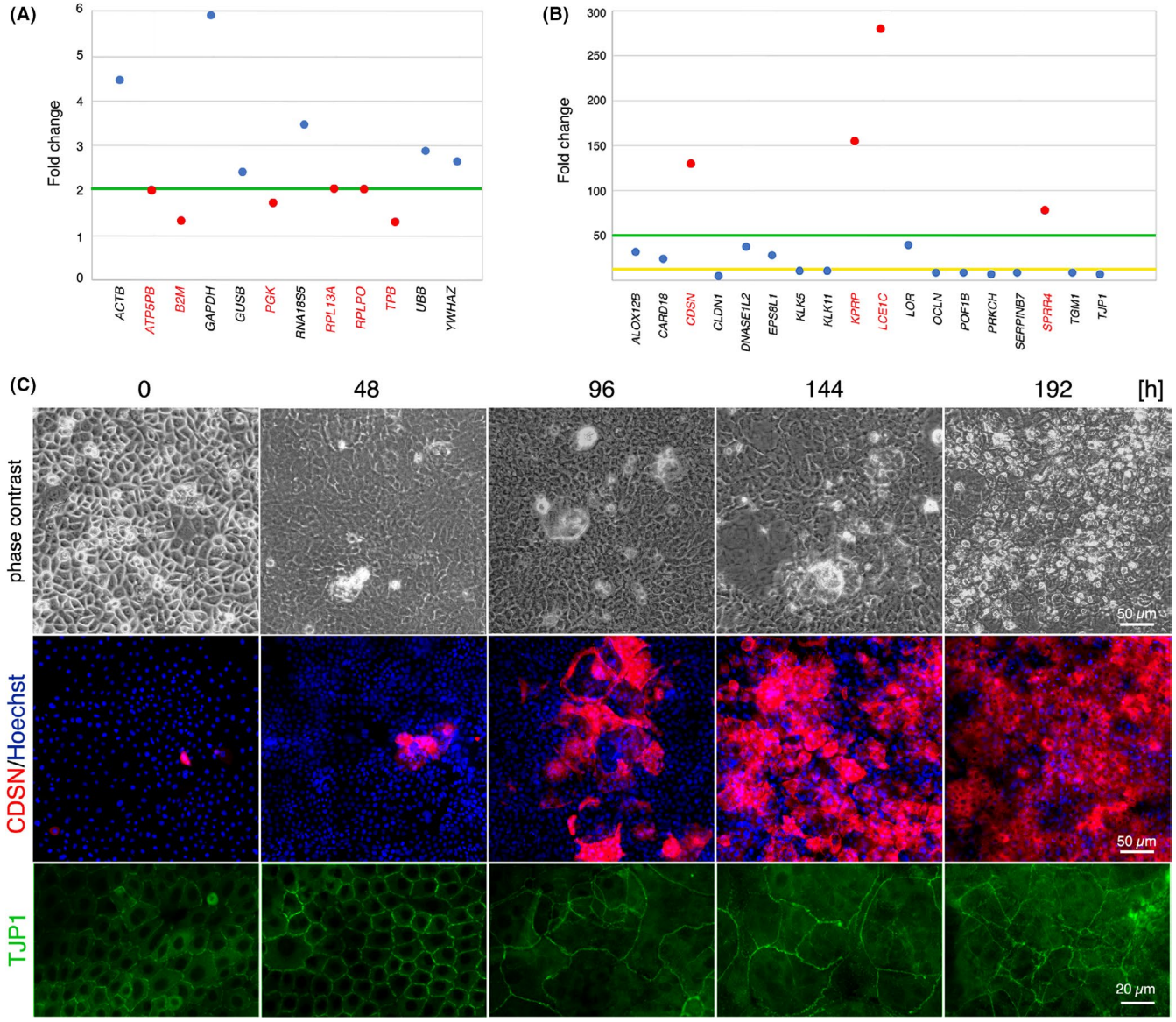

FIGURE 1 (A) Expression of the 12 candidate reference genes in proliferating keratinocytes (0 h) and following exposure to 1.5 mmol/L Ca^{++} for 48, 96, 144 and 192 h. Expression is presented as a fold change between gene copy number in standard keratinocyte culture that were not exposed to 1.5 mmol/L Ca^{++} and maximal change in gene copy number in those that were exposed to 1.5 mmol/L Ca^{++}. Among them, the six genes marked in red (*B2M*, *PGK1*, *TBP*, *RPLPO*, *RPL13A* and *ATP5BP*) were sufficiently stable (fold change, FC ≤ 2.00) during Ca^{++}-induced terminal differentiation of keratinocytes in vitro to serve as reference genes for all future RT-qPCR analyses. (B) Following the exposure to + 1.5 mmol/L Ca^{++} for 96-144 h, the expression of 18 genes known to be expressed in SG/SC and that we examined here, changed. Based on FC, the genes could be grouped into three clusters: <10-fold increase (below yellow line: *CLDN1*, *KLK5*, *KLK11*, *OCLN*, *POF1B*, *PRKCH*, *SERPINB7*, *TGM1* and *TJP1*), 10- to 50-fold (between yellow and red lines: *ALOX12B*, *CARD18*, *DNASE1L2*, *EPS8L1* and *LOR*), and >50-fold (above red line: *CDSN*, *KPRP*, *LCE1C* and *SPRR4*). For normalization, we used six reference genes that we determined earlier. (C) Phase contrast images of keratinocyte monolayer grown to confluence (0 h) and exposed to 1.5 mmol/L Ca^{++} for 48, 96, 144 and 192 h (upper panel). Expression of CDSN (mid panel, red) and TJP1 (lower panel) under given conditions. Blue in mid panel, nuclei stained with Hoechst 33342

TABLE 1 Ca^{++} induced gene expression of SG/SC markers in 2D cultures

Gene	Exp at 0 h [norm. mean copy number]	Max exp [norm. mean copy number]	Fold increase
ALOX12B	70 878	2 018 658	28.48
CARD18	4820	103 109	21.39
CDSN	2221	280 405	126.25
CLDN1	133 530	320 709	2.40
DNASE1L2	1727	59 649	34.54
EPS8L1	513	12 376	24.12
KLK5	7665	56 660	7.39
KLK11	683	5623	8.23
KPRP	32	4828	150.88
LCE1C	85	23 536	276.89
LOR	397	14 045	35.38
OCLN	1111	5986	5.39
POF1B	1403	8077	5.76
PRKCH	64	231	3.61
SERPINB7	9691	52 562	5.42
SPRR4	73	5398	73.95
TGM1	1463	6526	4.46
TJP1	213	818	3.84

Note: Genes with highest response to increase Ca^{++} level.Genes that had >50-fold increase in expression following exposure to 1.5 mmol/L Ca^{++} are highlighted with yellow background (*CDSN*, *KPRP*, *LCE1C*, *SPRR4*).

that in vitro conditions might cause aberrant expression of these genes and that the results could be easily misinterpreted. Similarly, in gene expression studies it is frequently overseen that the expression of some of reference/housekeeping genes might be sensitive to change in Ca^{++} concentration, which again might skew data interpretation.[11-14]

2 | QUESTION ADDRESSED

The objective of this study was to identify optimal RT-qPCR and immunostaining reference genes/markers for Ca^{++}-induced terminal differentiation of NHK in an in vitro 2D system.

3 | EXPERIMENTAL DESIGN

We cultured NHKs from a pool of five donors in animal product-free, chemically defined medium to confluency. Then, we exposed the cells to 1.5 mmol/L Ca^{++} for 48, 96, 144 or 192h in the same culture medium and analysed gene expression at mRNA and protein level.

To correctly interpret results from RT-qPCR, the data should be normalized using reference genes, which show minimum variation in the expression levels between different samples and experimental conditions. Since exposure to higher concentration of Ca^{++} might result in fluctuation of reference gene expression,[11-14] we screened 12 commonly used reference genes to choose the best ones for RT-qPCR analyses under these conditions. Next, using RT-qPCR and 6 reference genes with stable expression, we analysed the expression of the 18 known SG/SC markers. The findings were confirmed at protein level with immunostaining.

For complete materials and methods, see Appendix S1. Sequence of RT-qPCR primers for reference genes are listed in Table S1 and for SG/SC markers in Table S2.

4 | RESULTS

From 12 common reference genes that we analysed (*ACTB*, *ATP5PB*, *B2M*, *GUSB*, *PGK1*, *RNA18S5*, *RPL13A*, *RPLPO*, *TBP*, *UBB* and *YWHAZ*), we found that only 6 of them (*B2M*, *PGK1*, *TBP*, *RPLPO*, *RPL13A* and *ATP5BP*) were sufficiently stable during Ca^{++}-induced terminal differentiation of keratinocytes in vitro (Figure 1A). Results of geNorm analyses for the 6 most stable reference genes that were used in calculating the normalization factor are shown in Figure S1A. Expression data of all 12 reference genes analysed are shown in Figure S1B-M.

To determine which genes, considered as markers of the keratinocytes in the SG/SC,[13] have similar pattern of expression in vivo and in vitro following Ca^{++}-induced terminal differentiation, we analysed the expression of the following 18 known SG/SC markers: *ALOX12B*, *CARD18*, *CDSN*, *CLDN1*, *DNASE1L2*, *EPS8L1*, *KLK5*, *KLK11*, *KPRP*, *LCE1C*, *LOR*, *OCLN*, *OTX1*, *POF1B*, *PRKCH*, *SERPINB7*, *SPRR4*,

TGM1 and TJP1. For normalization, we used six reference genes that we determined earlier.

In comparison with dermal fibroblasts used as a negative control, and all SG/SC markers tested had shown some level of expression throughout all time points with *KPRP*, *LCE1C*, *PRKCH* and *SPRR4* having the lowest copy number (<100) and *ALOX12B* and *CLDN1* the highest (>50,000) (Table 1). All of them had a peak of expression between 96 and 144 hours. *CLDN1*, *KLK5*, *KLK11*, *OCLN*, *POF1B*, *PRKCH*, *SERPINB7*, *TGM1* and *TJP1* had only a moderate increase at the peak of their expression (<10-fold), which renders them unreliable as markers of Ca^{++}-induced terminal differentiation of keratinocytes in 2D cultures (Figure 1B). Their presence might be interpreted as the keratinocyte cultures already reached distinctive phenotype of outer layers of epidermis even though they did not. The best candidates seem to be SC markers *CDSN*, *KPRP*, *LCE1C* and *SPRR4*, which have the highest fold increase of their expression upon exposure to 1.5 mmol/L Ca^{++} (126.25, 150.88, 276.89 and 73.95-fold, respectively). Expression data of all 18 SG/SC marker genes analysed are shown in Figure S2A-R

To test whether protein expression correlates to our RT-qPCR data, for immunodetection we chose one of the top 4 candidates (*CDSN*) and one that did not show expected pattern in our RT-qPCR data set (*TJP1*) despite being widely used in published literature as proof of terminal differentiation (Figure 1C). Indeed, similarly to our RT-qPCR data, CDSN expression pattern recapitulated what is seen in vivo, whereas TJP1 did not. In vivo in healthy epidermis TJP1 is localized exclusively in SG, and however, in vitro TJP1 was expressed in nearly all keratinocytes, both proliferating and transdifferentiating.

5 | CONCLUSIONS

We determined that *B2M*, *PGK1*, *TBP*, *RPLPO*, *RPL13A* and *ATP5BP* are suitable reference genes for transcriptome analysis of Ca^{++}-induced terminal differentiation of keratinocytes in 2D cultures. We have also shown that under these conditions, SC markers *CDSN*, *KPRP*, *LCE1C* and *SPRR4* follow expression pattern in vivo and can be used as markers of Ca^{++}-induced terminal differentiation of keratinocytes in 2D cultures in vitro, whereas some commonly used markers such as *TJP1* may not be representative.

These 18 genes that we analysed represent only a subset of genes expressed in SG/SC. To make a more comprehensive analysis and take in consideration all or nearly all possible markers of keratinocyte terminal differentiation, RNAseq approach would be more suitable than qRT-PCR. However, RNAseq is much more expensive and time-consuming, which makes it unsuitable for a standard Quality Control of the experiment. The other genes might be more representative in different experimental settings (ie different source of keratinocytes, different culture medium) or different laboratories.

Our results suggest that in spite of its wide use, data obtained using 2D Ca^{++}-induced terminal differentiation of NHK should be subjected to additional scrutiny before conclusions could be made.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

AUTHOR CONTRIBUTIONS

DI, AC and TMM initiated the study and wrote the manuscript; MG isolated and prepared primary keratinocytes; JJ, NK and PK conducted experimental work.

DATA AVAILABILITY STATEMENT

Data available in article Appendix S1.

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SUPPORTING INFORMATION

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

Fig S1. Summary of data used in calculating the normalization factor of six reference genes

Fig S2. Summary of data used in calculating the expression of SG/SC marker genes

Tab S1. RT-qPCR primers for reference genes

Tab S2. RT-qPCR primers for genes expressed in SG and SC in vivo. The primers are designed as an intron-spanning assay. If genomic DNA is present in the sample will not be amplified

App S1. Material and methods

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