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Meaning of relative gene expression in multilayered cultures of epidermal keratinocytes

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Abstract

Reconstructed human epidermis (RHE) has become an in vitro model of choice for studying cell and tissue functions. Analysis of gene expression over the course of reconstruction must take into account the heterogeneous differentiation states of keratinocytes reconstituting the typical epidermal layers. In monolayer cultures, relative mRNA expression levels of differentiation markers are usually expressed as a ratio versus a classical reference gene (also named house-keeping gene) tested to be expressed equally in certain experimental conditions. Applied to complex tissues in which the cell number increases over time together with differentiation, calculation of relative gene expression does not take enough into account a crucial phenomenon: epidermal morphogenesis results in progressive restriction of differentiation markers, such as involucrin, to a specific layer, or in the delayed onset of mRNA expression of filaggrin or TMEM45A for instance following stratification. Our study illustrates that comparing the relative expression level of mRNAs to that of a basal layer-specific gene (e.g. ITGA6) better illustrates the contribution of specific differentiation markers to the process of epidermal morphogenesis.

Background

Several culture models are available for the study of epidermal keratinocytes, from monolayers to multilayered reconstructed tissues (1). Recently, the development of reconstructed human epidermis (RHE) from stratified cultures of keratinocytes produced at the air-liquid interface over a porous membrane (2,3) or on a dermal matrix (4) has enriched experimental approaches of epidermal functions, such as those linked to the barrier. However, the heterogeneous differentiation states of keratinocytes involved in stratification of the RHE, renders the analysis of gene expression more complicated than in monolayers.

For analysis of gene expression, specific messenger RNA are quantified using most often qRT-PCR. In order to account for the cell number in each sample, the expression level of the gene of interest is divided by the mRNA level of a reference gene, i.e. a gene considered as transcribed at a constant level in defined experimental conditions. Diverse

reference genes are proposed (5), including GAPDH (6), cytoskeletal components, or ribosomal genes (RPLP0 or RPL13a) (5-9). The choice of adequate reference gene is better based on experimental selection of the most stable candidate (5). Applied to autocrine keratinocyte monolayers undergoing differentiation, normalization of mRNA levels to RPL13a appears suitable. Indeed, the relative expression levels of most epidermal differentiation genes increase as confluence is attained, accurately reflecting the change in cell phenotype revealed by loss of proliferation and clonogenicity, together with initiation of cell stratification (9).

Question addressed

The purpose of this letter is to draw attention of researchers to take caution when analyzing gene expression in RHE with techniques developed in the context of homogeneous cell populations. In particular, we show that comparing the relative expression level of mRNAs encoding differentiation markers to that of a basal layer-specific gene better illustrates the development of the morphogenesis process.

Experimental design

This study is focused on analysis of the relative mRNA expression of several epidermal differentiation markers during *in vitro* morphogenesis of RHE, and comparison to the distribution of the corresponding proteins by immunohistochemistry (2). Procedures for RNA extraction (3), qRT-PCR (Supplementary method and Table 1) and calculation of relative mRNA expression by the ΔΔCt method were performed as described (5,8). The differentiation process was controlled with keratin 10 and involucrin staining (Figure S1).

Results

In order to assess whether RPL13a is an adequate reference gene in our experimental settings, the immunoreactivity of the RPL13a gene product was monitored during the

reconstruction process. The RPL13a signal appeared uniformly distributed at all times throughout the living layers of the RHE (Figure 1), reflecting a constant mRNA expression level in every cell confirmed by geNorm analysis (10)(Figure S2). In addition, we sought to compare the mRNA levels of the genes of interest to those of mRNA species expressed solely in the basal layer (keratin 14; KRT14 and alpha6 integrin subunit; ITGA6). Immunostaining of the corresponding proteins revealed homogeneous labelling of every cell of the basal layer over the entire reconstruction period (Figure 1).

In parallel to these immunolabelling results, the relative mRNA levels of the basal markers ITGA6 and KRT14 decreased in comparison with RPL13a, as expected from the increasing number of cells (respectively Figure 2a and 2b).

Using RPL13a as reference gene, the relative expression levels of KRT10 and loricrin (Figure 2c and 2d) increased during reconstruction of the epidermis, suggestive of the development of a differentiated cell compartment. However, the relative expression of FLG was nearly stable (Figure 2e) and the relative expression levels of KRT14, IVL and TGM clearly decreased over the same period (Figure 2b, 2f and 2g). Similar results were observed using the mean of TBP and RPLP0 expression as reference (Figure S3). At first glance, these observations sounded in discordance with the progressive reconstruction of correctly differentiated epidermal layers shown by Frankart et al. (2). However, one has to consider other interpretations considering the various distributions of differentiation markers.

Indeed, the immunolabelling signals of KRT10 or FLG, which are barely detectable before stratification of keratinocytes, appear in differentiating layers during tissue reconstruction (2). Conversely, the immunoreactivity of IVL, a differentiation marker expressed at a significant level on day 1, concentrates in RHE in upper spinous and granular layers. This progressive restriction of IVL protein distribution likely explains why its relative expression to RPL13a decreases over the duration of culture (Figure 2f).

On the other side, calculation of relative mRNA levels compared to basal markers KRT14 or ITGA6 resulted in higher ratios as compared to those calculated in regard of RPL13a. After normalization with ITGA6, the changes in mRNA levels over time faithfully illustrate the process of differentiation, which is less evident using KRT14 as a reference. As an example, calculation in regard of ITGA6 indicated that the relative expression of IVL remained rather constant (Figure 2f). Simultaneously, the relative expression of TGM increased (Figure 2g). Interestingly, analysis of a novel marker of the granular layer, TMEM45A, showed stable expression in regard of RPL13a, whilst its level increased over time in regard of ITGA6 (Figure 2h). This result is in accordance with the distribution of TMEM45A immunoreactivity, which is present at a low level on day 1 and progressively concentrates in the granular layer (Figure S1)(11). The kinetics of both TMEM45A expression level and protein profile are reminiscent of that of FLG and TGM, known markers of the granular layer.

Conclusions

In conclusion, the meaning of relative gene expression varies between one homogeneous cell population on one hand, and the heterogeneous population found in complex tissues on the other hand. Altogether, expression levels calculated in regard of the ITGA6 mRNA during RHE morphogenesis best illustrate the localized distribution of markers, challenging the classical meaning of reference gene.

Author contributions

JM and YP designed the study. JM, MH and AH performed the research. JM, YP and CL analysed the data. JM,YP and CL wrote the paper.

Conflict of interest

The authors state no conflict of interest

Acknowledgments

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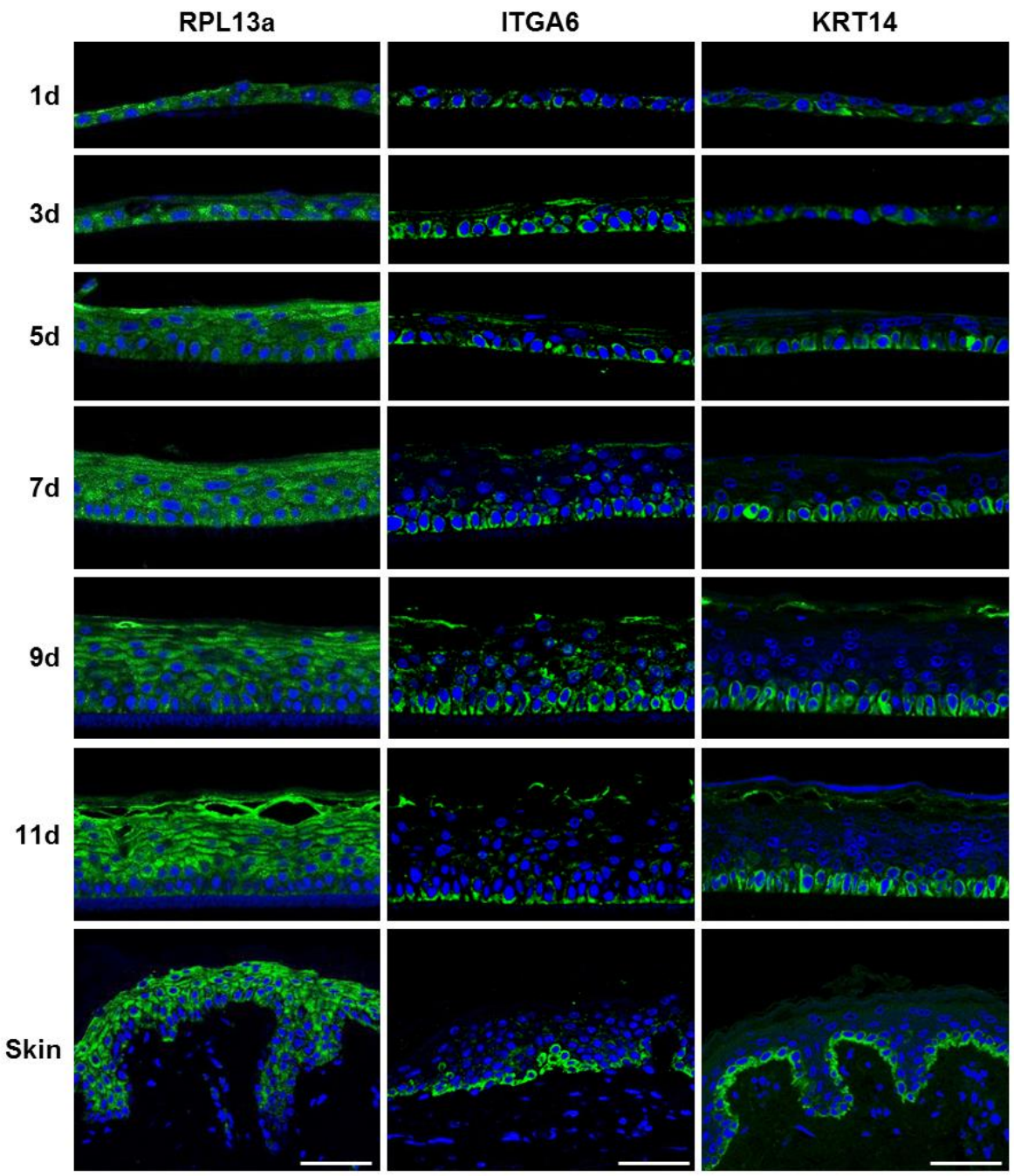
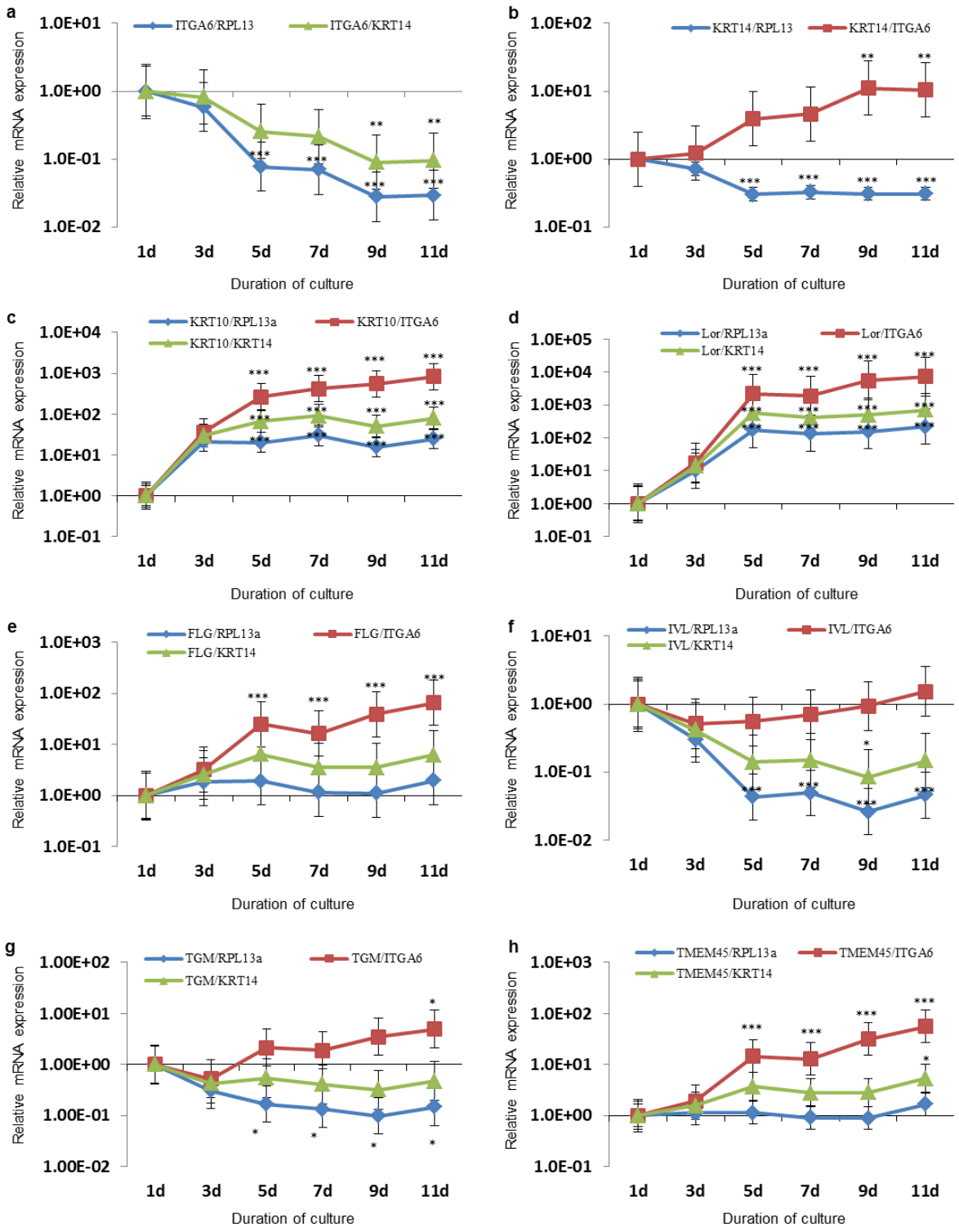
Figure 1: Localization of alpha6 integrin subunit, keratin 14 and RPL13a during reconstruction of an epidermis. Immunofluorescent staining of transversal histological sections of RHE and of normal skin. RHE were analyzed after 1, 3, 5, 7, 9 or 11 days of reconstruction. Primary antibodies against RPL13a, alpha6 integrin subunit (ITGA6), and keratin 14 (KRT14) were used, followed by Alexa 488-conjugated secondary antibodies (green). Nuclei are stained by Hoechst 33258 (blue). Sections were observed under confocal microscopy. Normal human skin is shown as control (bars = 50 µm).

Figure 2 : mRNA expression of differentiation markers normalized to keratin 14, alpha6 integrin subunit or RPL13a during the reconstruction of human epidermis. Total RNA was extracted from reconstructed human epidermis (RHE) after 1, 3, 5, 7, 9 or 11 days of growth.Reverse transcription was then performed and cDNA analyzed by real-time PCR. The expression levels of alpha 6 integrin subunit (ITGA6; a), keratin 14 (KRT14; b), keratin10 (KRT10; c), loricrin (Lor; d), filaggrin (FLG; e), involucrin (IVL; f), transglutaminase (TGM; g) and TMEM45A (h) were normalized to expression of RPL13a (blue), KRT14 (green), or ITGA6 (red). The mRNA level at day 1 was taken as reference and arbitrarily set at 1. The y-axis is a logarithmic scale and error bars represent 95% confidence intervals (n=3, ANOVA 1RM, *P<0.05, **P<0.01, ***P<0.001 vs 1d).


RPL13a
ITGA6
KRT14
1d
3d
5d
7d
9d
11d
Skin


a
ITGA6/RPL13
ITGA6/KRT14
Relative mRNA expression
1.0E+01
1.0E+00
1.0E-01
1.0E-02
1d 3d 5d 7d 9d 11d
Duration of culture
b
KRT14/RPL13
KRT14/ITGA6
Relative mRNA expression
1.0E+02
1.0E+01
1.0E+00
1.0E-01
1d 3d 5d 7d 9d 11d
Duration of culture
c
KRT10/RPL13a
KRT10/ITGA6
KRT10/KRT14
Relative mRNA expression
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
1.0E-01
1d 3d 5d 7d 9d 11d
Duration of culture
d
Lor/RPL13a
Lor/ITGA6
Lor/KRT14
Relative mRNA expression
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
1.0E-01
1d 3d 5d 7d 9d 11d
Duration of culture
e
FLG/RPL13a
FLG/ITGA6
FLG/KRT14
Relative mRNA expression
1.0E+03
1.0E+02
1.0E+01
1.0E+00
1.0E-01
1d 3d 5d 7d 9d 11d
Duration of culture
f
IVL/RPL13a
IVL/ITGA6
IVL/KRT14
Relative mRNA expression
1.0E+01
1.0E+00
1.0E-01
1.0E-02
1d 3d 5d 7d 9d 11d
Duration of culture
g
TGM/RPL13a
TGM/ITGA6
TGM/KRT14
Relative mRNA expression
1.00E+02
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1d 3d 5d 7d 9d 11d
Duration of culture
h
TMEM45/RPL13a
TMEM45/ITGA6
TMEM45/KRT14
Relative mRNA expression
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1.0E+02
1.0E+01
1.0E+00
1.0E-01
1d 3d 5d 7d 9d 11d
Duration of culture

Supplementary figures

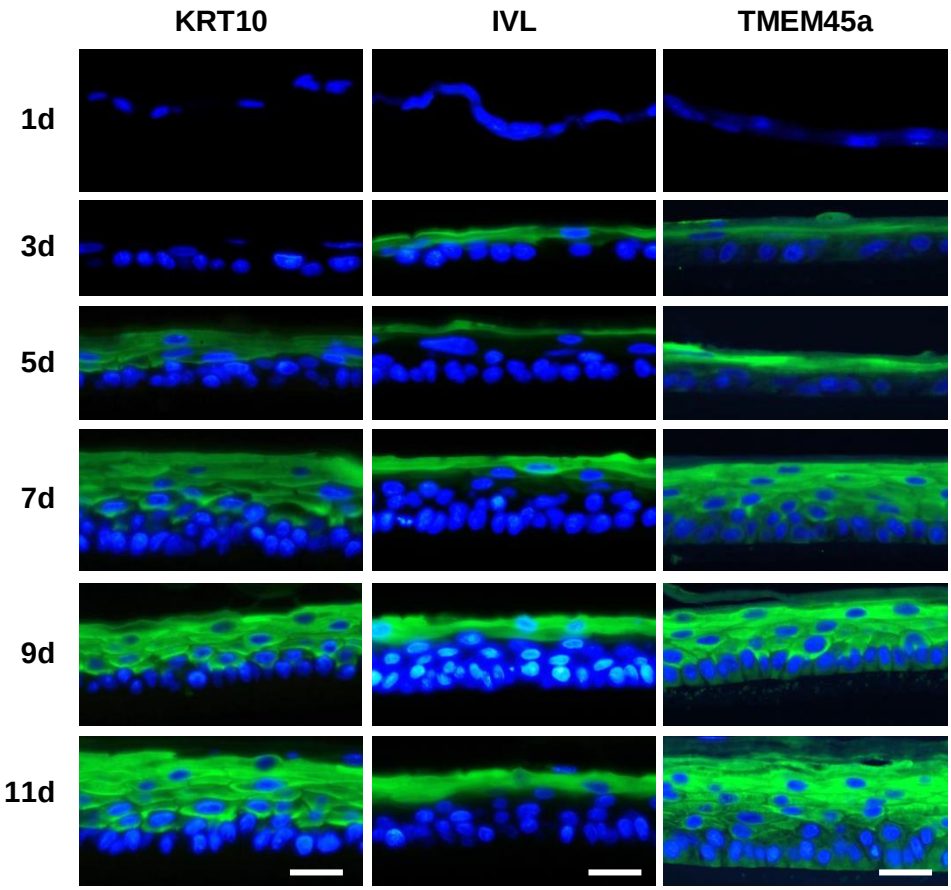

Figure S1: Localization of KRT10, IVL and TMEM45a during reconstruction of an epidermis. Immunofluorescent staining of transversal histological sections of RHE. RHE were analyzed after 1, 3, 5, 7, 9 or 11 days of reconstruction. Primary antibodies against keratin 10 (KRT10), involucrin (IVL), TMEM45a were used, followed by Alexa 488-conjugated secondary antibodies (green). Nuclei are stained by Hoechst 33258 (blue). Sections were observed under epifluorescence microscopy. (bars = 25 µm).

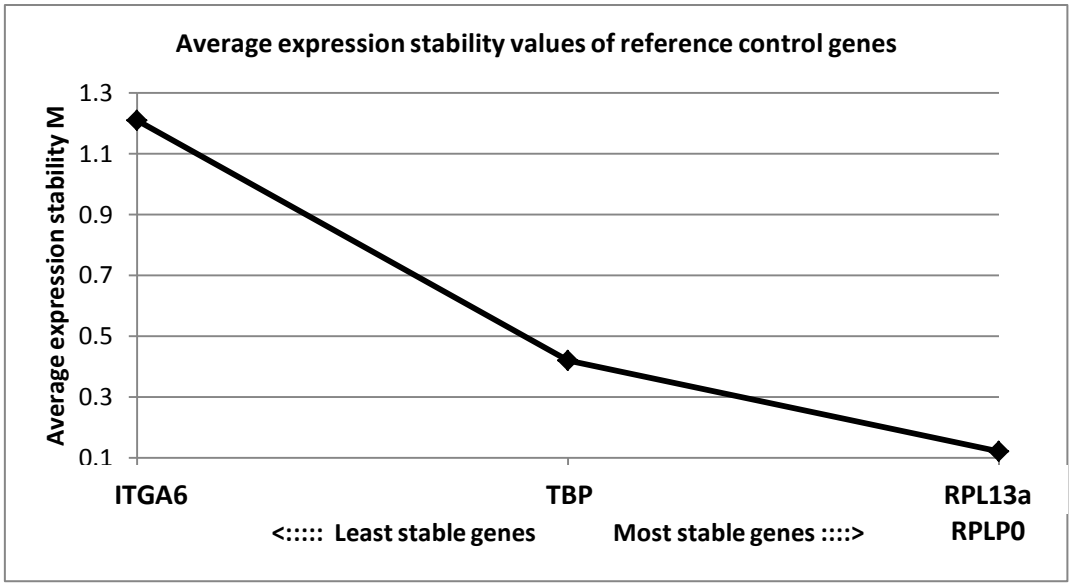

Figure S2 : Expression stability of reference genes (TBP-RPLP0-RPL13a) and basal layer reference gene (ITGA6). The geNorm analysis was performed in RHE at 1, 3, 5, 7, 9 or 11 days of reconstruction. Genes with the lowest M value have the most stable expression.

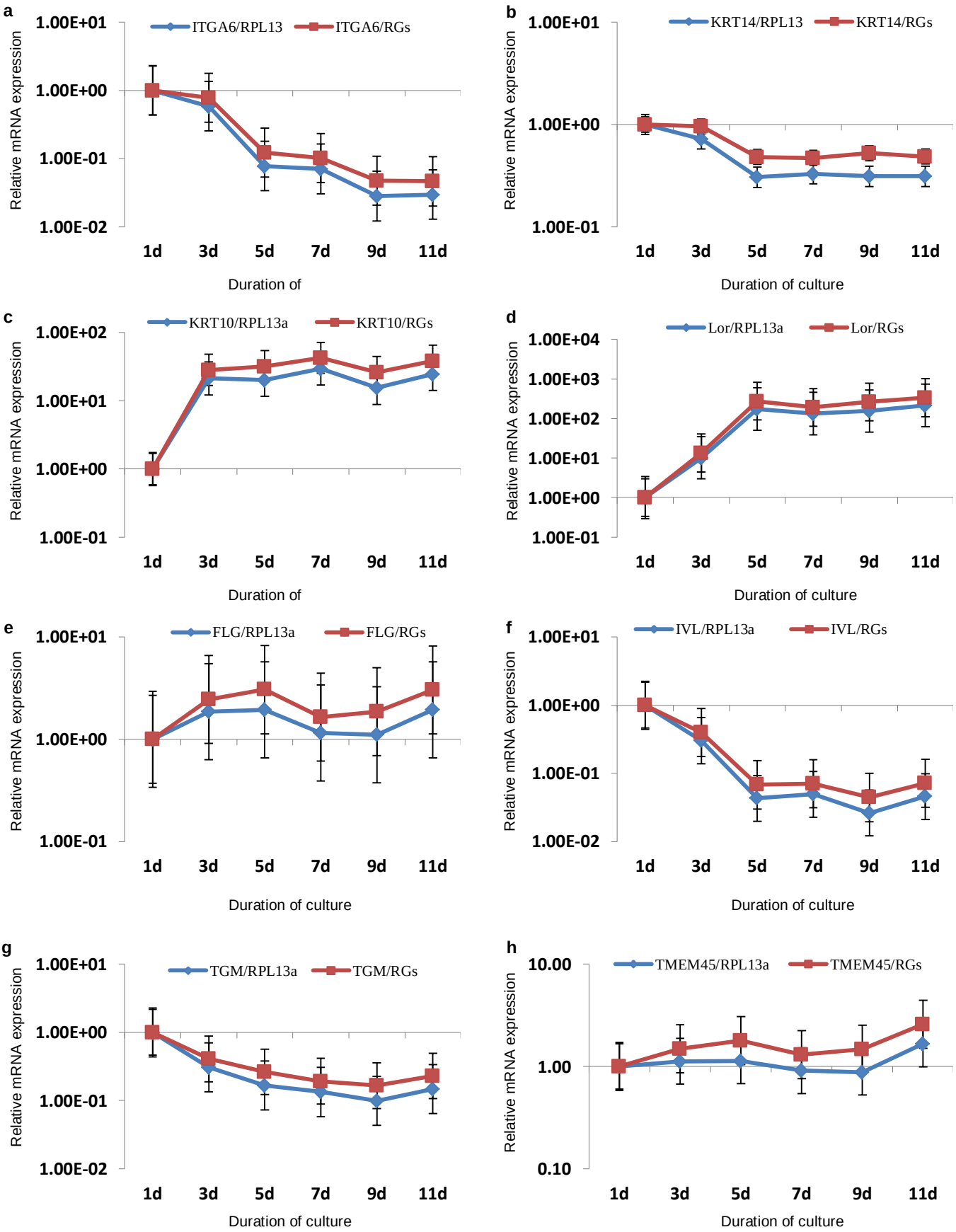
a
ITGA6/RPL13
ITGA6/RGs
Relative mRNA expression
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1d
3d
5d
7d
9d
11d
Duration of
b
KRT14/RPL13
KRT14/RGs
Relative mRNA expression
1.00E+01
1.00E+00
1.00E-01
1d
3d
5d
7d
9d
11d
Duration of culture
c
KRT10/RPL13a
KRT10/RGs
Relative mRNA expression
1.00E+02
1.00E+01
1.00E+00
1.00E-01
1d
3d
5d
7d
9d
11d
Duration of
d
Lor/RPL13a
Lor/RGs
Relative mRNA expression
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
1.00E-01
1d
3d
5d
7d
9d
11d
Duration of culture
e
FLG/RPL13a
FLG/RGs
Relative mRNA expression
1.00E+01
1.00E+00
1.00E-01
1d
3d
5d
7d
9d
11d
Duration of culture
f
IVL/RPL13a
IVL/RGs
Relative mRNA expression
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1d
3d
5d
7d
9d
11d
Duration of culture
g
TGM/RPL13a
TGM/RGs
Relative mRNA expression
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1d
3d
5d
7d
9d
11d
Duration of culture
h
TMEM45/RPL13a
TMEM45/RGs
Relative mRNA expression
10.00
1.00
0.10
1d
3d
5d
7d
9d
11d
Duration of culture

Figure S3: mRNA expression of differentiation markers normalized to RPL13a or mean of TBP and RPLP0 during the reconstruction of human epidermis. Total RNA was extracted from reconstructed human epidermis (RHE) after 1, 3, 5, 7, 9 or 11 days of growth.Reverse transcription was then performed and cDNA analyzed by real-time PCR. The expression levels of alpha 6 integrin subunit (ITGA6; a), keratin 14 (KRT14; b), keratin10 (KRT10; c), loricrin (Lor; d), filaggrin (FLG; e), involucrin (IVL; f), transglutaminase (TGM; g) and TMEM45A (h) were normalized to expression of RPL13a or mean of RPLP0 and TBP (RGs; red). The mRNA level at day 1 was taken as reference and arbitrarily set at 1. The y-axis is a logarithmic scale and error bars represent 95% confidence intervals (n=3).

Supplementary method

Total RNA isolation and relative-quantitative Real Time PCR

RNA was extracted using using RNeasy kit (Qiagen, Hilden, Germany) and concentration measured with NanoDrop 1000 (Thermo Scientific, Rockford, USA). Super Script II RNase H⁻reverse transcriptase (Invitrogen, Merelbeke, BE) was used for first strand cDNA synthesis. cDNA was amplified with the 7300 real-time PCR system (Applied Biosystems, Lennik, BE) after mixing with FastStart Universal SYBR Green Master (Roche, Basel, Switzerland). Primers (Eurogentec) were used at 300 nM. They are listed in Supplementary Table. Data were analyzed using the DDCT quantitative method (Livak and Schmittgen, 2001).

Table

Table 1. Sequences of primers

Gene symbol	Forward primer	Reverse primer
FLG	GGGCACTGAAAGGCAAAAAG	CACCATAATCATAATCTGCACTACCA
ITGA6	ATTCTCATGCGAGCCTTCAT	GGAAACACAGTCACTCGAACC
IVL	TGAAACAGCCAACTCCAC	TTCCTCTTGCTTTGATGGG
KRT10	AATCAGATTCTCAACCTAACAAC	CTCATCCAGCACCCTACG
KRT14	CGATGGCAAGGTGGTGTC	GGGTGAAGCAGGGTCCAG
LOR	TCATGATGCTACCCGAGGTTTG	CAGACCTAGATGCAGCCGGAGA
RPL13A	CTCAAGGTCGTGCGTCTGAA	TGGCTGTCACTGCCTGGTACT
TGM1	GTCGTCTTCCGGCTCGAA	TCACTGTTTCATTGCCTCCAAT
TMEM45A	TTATGCAGTAACCATTGTCATCGTT	TGATTCTTGTTCTCGTTCAGCATT