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Transcriptional activity of epigenetic remodelling genes declines in keratinocytes after *in vitro* expansion

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ABSTRACT

Purpose: In vitro expansion is an invaluable method to obtain keratinocytes in amounts necessary for effective transplantation therapies. *In vitro* cell culturing provokes questions concerning potential epigenetic alterations occurring in expanded cells in the context of usefulness for transplantation and safety. The purpose of this study was to investigate as to whether keratinocyte expansion is associated with changes in the activity of genes responsible for the maintenance of epigenetic stability.
Materials and methods: We focussed on the transcriptional activity of genes involved in different epigenetic mechanisms including DNA methylation and histone modifications. We used quantitative real-time PCR to determine transcript levels of 16 epigenetic remodelling markers in 14 patients in the epidermal cells directly after collection and after *in vitro* expansion.
Results: We observed a remarkable decline in the transcriptional activity of the epigenetic remodelling genes following *in vitro* expansion, while no further fall of expression with passaging. In whole skin, we found even higher expression levels of the epigenetic markers.
Conclusions: Transmission to *in vitro* environment challenges cellular signalling and metabolism. The regulation of epigenetic remodelling maintains the balance between cellular plasticity and phenotype deviation. This preliminary research demonstrated reduced activity of genes responsible for epigenetic modifications of DNA and histones in *in vitro* expanded epidermal cells. This observation indicates that epigenome re-patterning in cultured epidermal cells is significantly less intensive than in the skin. Also, this observation may imply that after adaptation to *in vitro* conditions, the epigenome does not undergo extensive transformation during further cultivation.

1. Introduction

Cell therapy based on autologous keratinocytes grafting is an effective method for the treatment of chronic wounds, severe burns and skin diseases. Transplantation of autologous cells is advantageous since there is no risk of graft rejection and viral transmission [1–4]. The success of this therapy depends mainly on the number of grafted cells, their viability, proliferative potential as well as delivery methods [5,6]. Transplanted keratinocytes stimulate wound healing through creating epidermis and secretion of growth factors and cytokines [1,7]. Keratinocytes may be delivered to patients in cell suspensions of freshly isolated cells or *in vitro* expanded cells, cultured epidermis or living skin equivalents [3,8,9]. The influence of culture conditions and passaging on regenerative potential of keratinocytes is poorly recognized. Therefore, comparing expanded keratinocytes with epidermal cells im-

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mediately after isolation would have important implications. Epigenetic status has been associated with skin diseases [10,11] and regenerative abilities [12–14]. Despite the importance of cell culture technologies in research and medicine, the question of epigenetic changes in cell cultures following tissue isolation has been understudied.

Exploring epigenetic processes directly in collected tissues is problematic, therefore, most studies concentrate on cultured cells. The aim of the presented study is to address the question of how the transfer of epidermal cells from *in vivo* to *in vitro* conditions affects epigenetic remodelling. Transcriptional profiling is an effective solution to carry out pioneering research into the processes of epigenetic remodelling. In this study, we selected a panel of 16 genes involved in DNA methylation and demethylation as well as histone modifications in order to determine their expression levels in human epidermal cells before and after *in vitro* expansion.

2. Patients and methods

2.1. Study groups

Skin samples were collected during surgery from 28 oncological patients suffering from gastrointestinal non-metastatic cancers (without chemotherapy and radiotherapy). The samples for keratinocyte expansion were collected from 7 female and 7 male patients, at the average and median age of 57 and 63 years, respectively. The tissues for direct RNA extraction from the whole-skin samples, were collected from 6 female and 8 male patients, at the average and median age of 66 and 67 years, respectively. The description of study groups is presented in Table 1.

2.2. Expansion of keratinocytes

A detailed protocol for keratinocyte expansion has been previously described [2]. Briefly, after collection, skin samples were treated with dispase (12U/mL, Corning, cat. no. 354235) in 37 °C for 2 h in order to separate the epidermis and to remove hair follicles. The separated epidermis was digested in 0.25% Trypsin/EDTA (Sigma-Aldrich, cat. no. T4049) in 37 °C for 10 min. An additional, 5 min trypsinization was applied, before digestion was stopped with trypsin inhibitor (Sigma-Aldrich, cat. no. T7659). Cells were washed in phosphate-buffered saline (Sigma-Aldrich, cat. no. D8537) containing penicillin and streptomycin (Sigma-Aldrich, cat. no. P4333), collected by centrifugation at 1700 rpm for 5 min and seeded on plastic dishes (25,000/cm2) following 24 h cultivation in humidified atmosphere with 5% CO_2 at 37 °C in keratinocytes serum-free growth medium (KBM Basal Medium, Lonza, cat. no. CC-3101) supplemented with epidermal growth factor, hydrocortisone, transferrin, epinephrine, insulin and gentamycin (SingleQuots Supplements pack, Lonza, cat. no. CC-4152). The medium was changed every second day. Cells were cultured until they reached confluency of 70–80%. For a passage, the monolayer was trypsinized, cells were centrifuged at 1700 rpm for 5 min, and seeded as above. Microscopic analyses were performed to estimate potential admixture of contaminating cells (melanocytes, Langerhans cells). Cells were collected by centrifugation and kept frozen until RNA isolation.

Table 1
The description of study groups.

Study groups					
EC group			WS group		
Patient	Sex	Age (years)	Patient	Sex	Age (years)
1	F	33	1	**F**	38
2	F	35	2	F	47
3	F	41	3	M	60
4	F	53	4	**F**	60
5	M	53	5	**F**	60
6	M	58	6	M	63
7	F	63	7	M	65
8	M	63	8	M	68
9	F	63	9	M	68
10	F	63	10	M	68
11	M	64	11	**F**	73
12	M	64	12	F	80
13	M	69	13	M	84
14	M	72	14	M	84

EC – isolated epidermal cells and cultured keratinocytes; WS – whole skin samples; F/M – female/male.

2.3. Processing of whole skin samples for direct RNA extraction

Immediately after collection, skin samples were transferred to RNAlater reagent (Qiagen) to prevent RNA degradation and stored at −80 °C.

2.4. Quantitation of transcript levels

RNA from isolated and cultured cells and from whole skin was extracted using an RNeasy Mini Kit and RNeasy Fibrous Tissue Mini Kit (Qiagen), respectively. cDNA synthesis was carried out in a final volume of 20 μL containing 100 pmoles of oligo dT20, 4 μL of 5x reaction buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$, 50 mM dithiothreitol), 200 units of Maxima Reverse Transcriptase (ThermoScientific) and 200 ng of RNA. Real-time PCR was performed in a final volume of 10 μL containing 5 μL of FastStart Essential DNA Green Master (Roche, Cat. No. 06402712001), 1 μL of cDNA, and 0.25 μL each of forward and reverse primers (10 μM) on a LightCycler LC96 (Roche). Expression levels were determined using the $2^{-\Delta\Delta Ct}$ method relative to three reference genes *Actb*, *Gapdh* and *Tbp*. PCR primers are listed in Table 2. PCR was performed in triplicate.

2.5. Statistical analyses

Statistical significance of the differences in gene expression was determined at $p < 0.05$ using the non-parametric Wilcoxon paired test. Correlation analyses were carried out using Pearson's method. Computations were performed in XLSTAT (Addinsoft).

2.6. Ethical issues

The procedures were approved by the Independent Bioethics Commission for Research at the Medical University of Gdańsk, Poland (NKEBN/359/2017, Issued 2007.09.24; NKBBN/547/2011–2012, Issued: 2012.03.27).

The study was conducted in accordance with the 1964 Declaration of Helsinki and its later amendments.

3. Results

3.1. Expression of epigenetic remodelling genes in epidermal cells in vitro before and after expansion

The transcript levels of 16 selected epigenetic markers were determined in epidermal cells directly after isolation and in two passages of cultured keratinocytes for 14 patients. The panel of analysed genes included those responsible for DNA methylation and demethylation, histone modifications and other involved in chromatin remodelling (Table 3). Gene expression differences between the population of freshly isolated epidermal cells, consisting mostly of keratinocytes (ISO), and the populations of cultured keratinocytes (P1, P2) were determined as fold

Table 2
PCR primer nucleotide sequences.

GENE	Forward primer	Reverse primer	Amplicon size bp
ACTB	CATGGGTCAGAAGGATTCCT	ACACGCAGCTCATTGTAGAA	150
AURKA	CACCTTCGGCATCCTAATATTCTT	GGGCATTTGCCAATTCTGTT	172
DNMT1	GAGCTACCACGCAGACATCA	CGAGGAAGTAGAAGCGGTTG	161
DNMT3A	TATTGATGAGCGCACAAGAGAGC	GGGTGTTCCAGGGTAACATTGAG	111
DNMT3B	CCCATTCGAGTCCTGTCATT	GGTTCCAACAGCAATGGACT	126
GAPDH	TGCACCACCAACTGCTTAG	GATGCAGGGATGATGTTC	176
HAT1	GTTATACTATATTGCTGGTAGCCT	ATCAACTTCCTTTTCCAGTAAAGA	181
HDAC3	TTGAGTTCTGCTCGCGTTACA	CCCAGTTAATGGCAATATCACAGAT	87
HDAC4	AATCTGAACCACTGCATTTCCA	GGTGGTTATAGGAGGTCGACACT	115
KAT6A	CAAACTGGTTGCACGAGAGA	CACAAGCACCTCTCCTGTGA	148
KDM1A	TTCTGGAGGGTATGGAGACG	ACCTTCTGGGTCTGTTGTGG	141
KDM4A	AGAGTTCCGCAAGATAGCCAA	AGTCCAGGATTGTTCTCAGCC	191
SUV39H2	ATCCCACCTGGTACTCCCATCT	GCAAAGCGAATACTGTGTGCC	102
TBP	TGCACAGGAGCCAAGAGTGAA	CACATCACAGCTCCCCACCA	132
TDG	AAGATGTGCTCAGTTTCCTCG	TAACAGCCATCTTCTTTGC	167
TET1	CAGAACCTAAACCACCCGTG	TGCTTCGTAGCGCCATTGTAA	141
TET2	GATAGAACCAACCATGTTGAGGG	TGGAGCTTTGTAGCCAGAGGT	95
TET3	TCCAGCAACTCCTAGAACTGAG	AGGCCGCTTGAATACTGACTG	169
ZMYND8	CAGAAAATGAAACAGCCAGGG	ACTTTGCATCAGCCAGGAAG	169

changes for each gene (ISO/P1, ISO/P2) and for each patient. Minimum twofold decreases in mean and median expression were found for all genes under the analysis in both passages (P1, P2) as compared to freshly isolated cells (ISO), and most differences were statistically significant (Fig. 1a,b). Remarkable, at least twofold differences in gene expression between the freshly isolated and cultured cells were observed for the predominant number of patients. For five genes: *TET1, TET2, TET3, KAT6A,* and *HDAC4,* the mean differences reached one order of magnitude. An analogous analysis performed to compare the first and second passage (P1/P2) did not reveal substantial changes (Fig. 1c).

3.2. Expression of epigenetic remodelling genes in whole skin samples

Elevated expression of epigenetic remodelling genes in freshly isolated epidermal cells relative to cultured keratinocytes could be explained by transient induction after the transition to *in vitro* environment followed by transcriptional decline during cultivation. Alternatively, the expression levels of epigenetic remodelling genes in the freshly isolated epidermal cells may reflect relatively high transcriptional activity in the skin. We performed transcriptional profiling in the whole skin samples directly after collection, and we found markedly higher expression of the analysed markers than in either the freshly isolated and or the cultured keratinocytes (Fig. 2), which indicated the potential effect of culture conditions.

3.3. Analysis of correlations between the age of patients and transcript levels

The age of tissue donors is known to have a powerful impact on the properties of cultured cells [15]. The age of patients in the study group ranged from 33 to 72 years (Table 1). To estimate the potential impact of patients' age on our gene expression results, we calculated Pearson coefficients of correlation between the age of patients and transcript levels. We found no meaningful correlations between these variables, in both freshly isolated and passaged cells (Table 4). A single exception was the marginally significant inverse correlation of 58% between the *HDAC3* expression and the age of patients in the freshly isolated epidermal cells ($p = 0.031$).

4. Discussion

Transcriptional profiling showed a significantly higher expression of epigenetic remodelling genes in freshly isolated epidermal cells in comparison with expanded keratinocytes cultured *in vitro* (Fig. 1). Even higher expression levels of the epigenetic markers were observed in whole skin samples (Fig. 2). Interestingly, the activated genes were responsible for counter-acting functions such as DNA methylation and DNA demethylation, histone acetylation and histone deacetylation (Table 3). The observed changes in gene expression could account for selective adherence of cells of certain phenotypes to plastic dishes but they may also be explained by the impact of culture conditions. However, it should be stressed that no sorting was performed to enrich the population of freshly isolated epidermal cells in stem cells.

The decreased activity of epigenetic remodelling genes in cultured keratinocytes may suggest a lower plasticity of cultivated cells, and, in turn, reduced capabilities for adaptation. However, our previous study [2] demonstrated that keratinocytes passaged in the same culture conditions as described in the present study maintained the ability for successful transplantation into the wound. The cultured keratinocytes displayed enrichment in CD49f (integrin alpha 6) and CD29 (integrin beta 1) but they did not represent the CD49f high and CD71 low (transferrin receptor) profile which is characteristic of keratinocyte stem cells [2]. It should be noted that the RNA templates isolated in the previous work [2] were used in the present study. Epigenetic regulation is connected with cellular plasticity but also cancer transformation [16,17]. To point out the importance of epigenetic remodelling in this context, it is worth noting that the inhibition of KAT6A, a histone acetyltransferase, one of the markers examined in the present study, was reported to induce senescence in cancer cells and arrest of tumour growth [18].

The decline in expression of genes participating in epigenetic modifications of DNA and histones in *in vitro* expanded keratinocytes indicated that after the adaptation to *in vitro* conditions the epigenetic remodelling was suppressed. The finding that the lowered activity of epigenetic remodelling genes was determined in two passages suggested that after adaptation to *in vitro* conditions, the epigenome did not go through extensive changes during cell culture. Certainly, this point requires large-scale experimental epigenome exploration. However, the examined genes are responsible not only for modifying the epigenome but also for its maintenance and reversion of transient epigenetic changes. In this aspect, further research is needed to investigate if the reduced activity of the genes involved in epigenetic processes may lead to a decreased potential to restore the epigenetic pattern.

It is important to consider the possible impact of experimental conditions, in the first place, the age of patients and cell culture medium. It is difficult to predict the levels of epigenetic markers for much younger and much older patients than those in the study group aged

Table 3
Epigenetic remodelling markers under the study.

Gene	Full name	Function	Category
DNMT1	DNA methyltransferase 1	Maintaining methylation patterns following DNA replication	DNA methylation
DNMT3A	DNA methyltransferase 3 alpha	*De novo* methyltransferases involved in the establishment of genomic methylation patterns in development	
DNMT3B	DNA methyltransferase 3 beta		
TET1	tet methylcytosine dioxygenase 1	Dioxygenases which catalyse the successive oxidation of 5-methylcytosine to 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine leading to active DNA demethylation.	DNA demethylation
TET	tet methylcytosine dioxygenase 2		
TET3	tet methylcytosine dioxygenase 3		
TDG	thymine DNA glycosylase	Mediates BER excision of 5-formylcytosine and 5-carboxylcytosine thus playing a key role in active DNA demethylation.	
KAT6A	lysine acetyltransferase 6A	Transfer of acetyl group on Lys of histones	Histone acetylation
HAT1	histone acetyltransferase 1 × 1		
HDAC3	histone deacetylase 3	Deacetylation of Lys on the N-terminal part of histones	Histone deacetylation
HDAC4	histone deacetylase 4		
SUV39H2	suppressor of variegation 3-9 homolog 2	Trimethylation of Lys-9 of histone H3	Histone methylation
KDM1A	lysine demethylase 1A	Demethylation of mono- and di-methylated Lys-4 of histone 3 (H3K4me)	Histone demethylation
KDM4A	lysine demethylase 4A	Specific demethylation of Lys-9 and Lys-36 of histone H3	
ZMYND8	zinc finger MYND-type containing 8	Receptor for activated C-kinase (RACK); contains a bromodomain involved in the recognition of acetylated histones	Regulation of chromatin remodelling
AURKA	aurora kinase A	Cell cycle-regulated kinase; plays critical roles in various mitotic events.	

from 33 to 72 (Table 1). However, it is worth noting that we did not find meaningful correlations between the age and transcriptional activity of the examined genes, thus indicating that the decline of epigenetic remodelling in cultured keratinocytes was not dependent on the age of tissue donors. The role of cell culture medium could be critical. We used a medium dedicated for transplantation applications. The medium is supplemented with growth factors to support proliferation but it is serum free to prevent cell differentiation [19–23]. It does not seem unlikely that the use of this type of culture medium was decisive to suppress the epigenetic remodelling.

Our observations add to the discussion on safety concerns regarding transplantation of cultured cells. Another important implication relates to pharmacological and cosmetics research, where cell culture models are widely employed. Epigenetic changes can lead to alterations in cel-

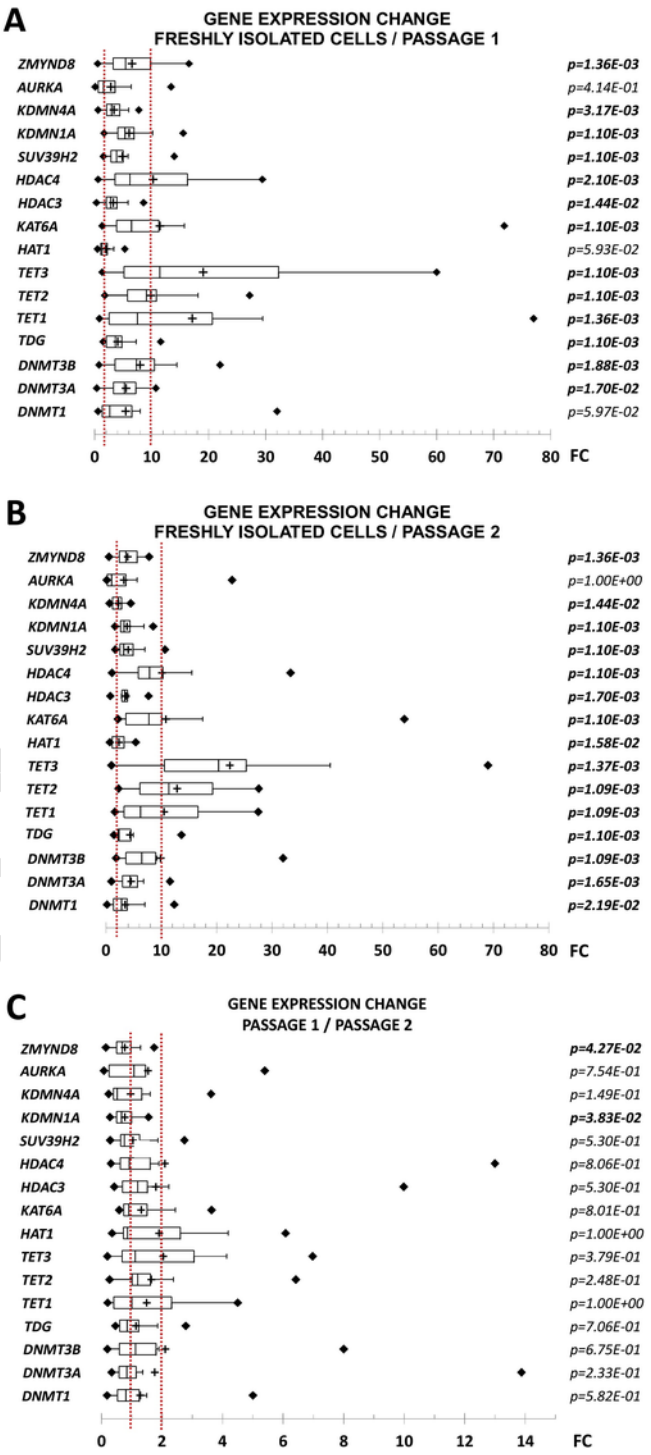

Fig. 1. Epigenetic remodelling genes show decreased transcriptional activity in keratinocytes after *in vitro* expansion.The differences in gene expression between freshly isolated epidermal cells (ISO) and passages of cultured keratinocytes (P1, P2) were calculated as fold changes (FC) for individual patients. The diamond symbols ♦ indicate maximal and minimal values of fold change determined for a given patient and transcript; the boxes indicate the first to the third quartile range: + and – marks in the boxes indicate the means and medians, respectively; the error bars correspond to standard deviation. The red dotted lines indicate 2-10-fold changes in expression in the panels A and B, and 1-2-fold changes in expression in the panel C.

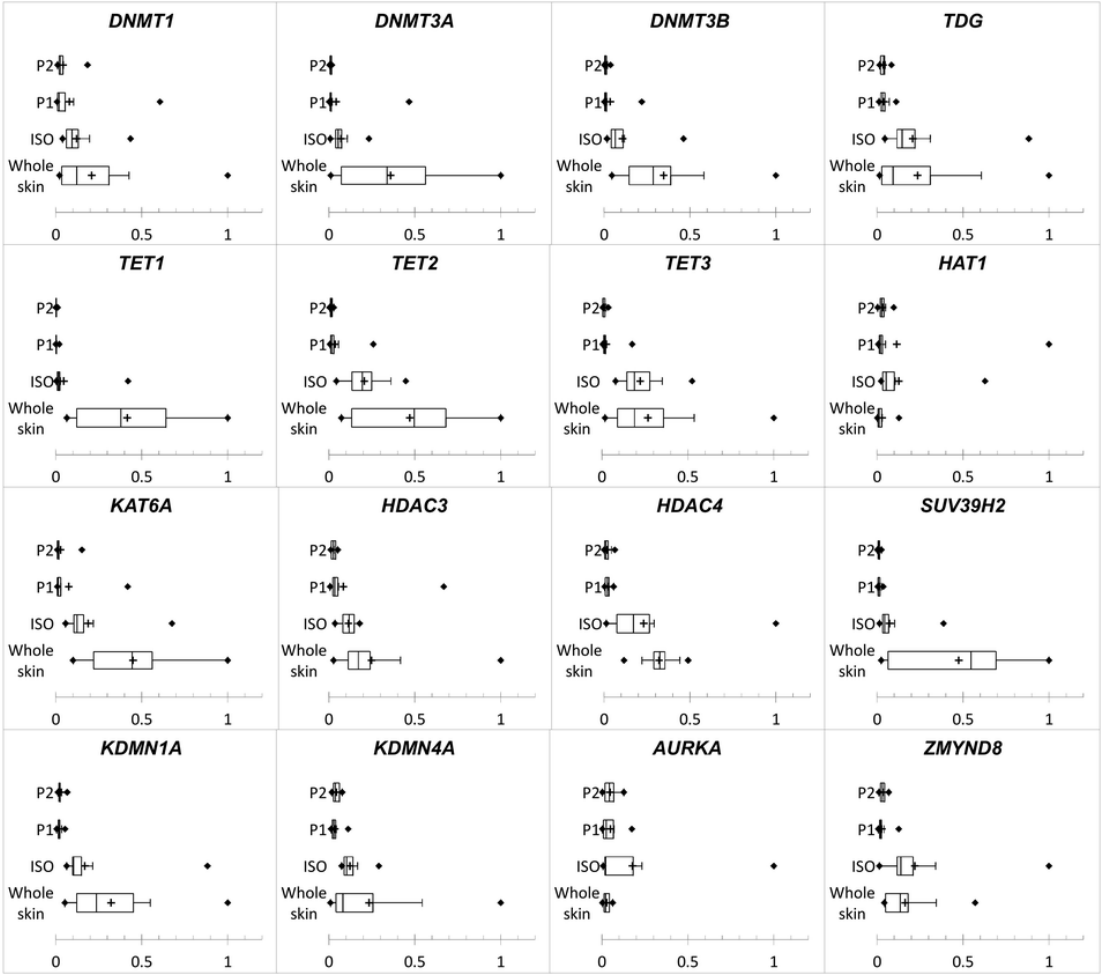

Fig. 2. Epigenetic remodelling genes show higher expression levels in the whole skin and freshly isolated epidermal cells than in *in vitro* expanded keratinocytes.The results represent relative normalized expression. The transcript levels were normalized for each gene to the highest value (1.0). The box plot symbols are explained in the legend to Fig. 1.ISO – freshly isolated epidermal cells; P1 – cultured keratinocytes passage 1; P2 – cultured keratinocytes passage 2; WS - whole skin

Table 4
Correlations between transcript levels and age of patients.

CORRELATION WITH AGE			
GENE	ISO	P1	P2
DNMT1	−0.268	0.255	0.027
DNMT3A	−0.400	0.164	−0.298
DNMT3B	0.067	0.279	−0.046
TDG	0.324	−0.111	0.023
TET1	−0.078	−0.138	−0.056
TET2	−0.215	0.206	−0.075
TET3	−0.259	−0.141	−0.060
HAT1	0.153	0.205	0.323
KAT6A	0.037	0.239	0.137
HDAC3	**−0.575**	0.067	−0.284
HDAC4	−0.159	−0.016	−0.177
SUV39H2	−0.133	−0.107	0.159
KDMN1A	0.229	0.387	0.207
KDMN4A	0.031	0.491	−0.095
AURKA	0.335	0.359	0.459
ZMYND8	−0.162	0.320	−0.326

The correlation coefficients were determined using Pearson's method. The significant results are bolded.
ISO – freshly isolated epidermal cells; P1 – cultured keratinocyte passage 1; P2 – cultured keratinocytes passage.

lular responses to tested compounds [24,25], while transcriptional profiling of epigenetic activity may help include such interference.

5. Conclusions

We report preliminary data showing a remarkable decrease in transcriptional activity of genes involved in DNA methylation and histone modifications in keratinocytes following *in vitro* expansion as compared to freshly isolated epidermal cells. The results indicated that the expanded keratinocytes did not show dynamic epigenetic remodelling during culturing. The repression of epigenetic remodelling genes appears to be an important feature of keratinocytes in *in vitro* conditions. The finding sheds light on functioning of cell culture models in various aspects such as cellular plasticity, adaptation and responses to drugs.

Conflict of interests

The authors declare no conflict of interests.

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Data statement

The data files underlying the study will be available at request.

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