KNOCKOUT OF MUTANT *TP53* IN THE HACAT CELLS ENHANCES THEIR MIGRATION ACTIVITY

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The HaCaT cell line represents the spontaneously immortalized non-carcinogenic human keratinocytes that are used as a model for studying the function of normal human keratinocytes. There are two *TP53* alleles in the HaCaT cell genome, which comprise two gain-of-function (GOF) mutations acquired through spontaneous immortalization (*mutTP53*). Mutations result in the increased proliferation rate and violation of the stratification program. The study was aimed to assess the effects of the *mutTP53* gene knockout on the HaCaT keratinocytes capability of proliferation and migration in the in vitro model of epidermal injury and regeneration (scratch test), and on the ability to form stratified epithelium in the organotypic epidermal model. To perform the scratch-test, cells were cultured until monolayer was formed, then the standardized wound was created. The organotypic model was obtained by growing keratinocytes in the polycarbonate membrane inserts with the pore size of 0.4 μm at the interface between the phases (air-liquid). It has been shown that the mutant *TP53* gene knockout results in the increased migration capability of the HaCaT keratinocytes: in the *mutTP53* knockout HaCaT, the defect closure occurred faster than in the appropriate group of the WT HaCaT ($p < 0.05$), on day three the defect size was 12% ± 3% and 66% ± 5% of the initial size. There is evidence that mutant *TP53* in the HaCaT cells is a negative regulator of the laminin-5 expression (*LAMC2* expression was 9.96 ± 1.92 times higher in the cells with the *mutTP53* knockout, $p < 0.05$), however, this does not promote normalization of the program of epithelial differentiation and stratification followed by formation of the stratum corneum in the organotypic model.

Keywords: HaCaT, keratinocyte differentiation, p53, CRISPR/Cas9, knockout, migration

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НОКАУТ МУТАНТНОГО *TP53* В КЛЕТКАХ ЛИНИИ НАСАТ УСИЛИВАЕТ ИХ МИГРАЦИОННУЮ АКТИВНОСТЬ

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Линия HaCaT — спонтанно иммортализованные неканцерогенные кератиноциты человека, широко используемые в качестве модели для изучения функций нормальных кератиноцитов человека. В геноме клеток HaCaT присутствуют две аллели гена *TP53*, которые содержат две gain-of-function (GOF) мутации, приобретенные в результате спонтанной иммортализации (*mutTP53*). Наличие мутаций приводит к увеличению скорости пролиферации и нарушению программы стратификации. Целью исследования было изучить влияние нокаута гена *mutTP53* на способность кератиноцитов линии HaCaT к пролиферации и миграции в модели повреждения и регенерации эпидермиса *in vitro* (скретч-тест), а также на способность формировать многослойный эпителий в органотипической модели эпидермиса. Для проведения скретч-теста клетки культивировали до образования монослоя, затем наносили стандартизованное повреждение. Органотипическую модель получали культивированием кератиноцитов в поликарбонатных мембранных вставках с диаметром пор 0,4 мкм на границе раздела фаз (воздух-жидкость). Продемонстрировано, что нокаут мутантного гена *TP53* приводит к увеличению миграционной способности кератиноцитов линии HaCaT: для HaCaT с нокаутом mutTP53 закрытие дефекта происходило быстрее по сравнению с соответствующей группой WT HaCaT ($p < 0,05$), на третьи сутки размер дефекта составлял 12% ± 3% и 66% ± 5% от первоначального. Получены данные, что мутантный *TP53* в клетках HaCaT является негативным регулятором экспрессии ламинина-5 (экспрессия *LAMC2* была выше в клетках с нокаутом *mutTP53* в 9,96 ± 1,92 раз, $p < 0,05$), однако это не способствует нормализации программы дифференцировки и стратификации эпителия с образованием рогового слоя в органотипической модели.

Ключевые слова: HaCaT, дифференцировка кератиноцитов, p53, CRISPR/Cas9, нокаут, миграция

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The HaCaT cell line (spontaneously immortalized non-carcinogenic human keratinocytes) is widely considered a model for studying the function of normal human keratinocytes [1, 2]. However, stratification program and differentiation marker expression are abnormal in the HaCaT line keratinocytes [3].

It is known that there are two alleles of the *TP53* gene the HaCaT keratinocyte genome that comprise two gain-of-function (GOF) mutations acquired via spontaneous immortalization (H179Y and R282Q) [4]. *MutTP53* exerts significant effects on the increase in proliferation rate and cell growth in the HaCaT

cell line and has more than 7000 DNA binding sites. Furthermore, protein functions associated with apoptosis triggering are preserved [5]. It is known that mutant p53 functions in at least two ways: it affects the function of p63/p73 inhibiting their binding to DNA [6] or binds to new DNA sites via interaction with other transcription factors (NF-Y, E2F1, NF-KB, VDR, p63) [7, 8].

Among fundamental physiological characteristics of the HaCaT cells, the most interesting are as follows: proliferation, migration, stratification, and creation of three-dimensional organotypic structures.

We have shown previously that alterations in mutp53 activity in the HaCaT cells result in the altered expression of differentiation markers (such as caspase 14, involucrin and transglutaminase 1) and are associated with altered expression of p63 [9].

Understanding the features of the HaCaT cell physiology and underlying mechanisms is essential for assessment of limitations associated with using such cells as model ones. Moreover, studying cells containing *mutTP53* makes it possible to obtain new data for carcinogenesis studies [10, 11].

The study was aimed to assess the features of alterations in migration capabilities of the *mutTP53* knockout HaCaT cells.

METHODS

Cell lines and culture conditions

The HaCaT cell line was acquired from the cell culture collection of the German Cancer Research Center (DKFZ, Heidelberg; Germany). The cell culture of the *mutTP53* knockout (dp53) HaCaT keratinocytes was obtained earlier [13]. The cells were cultured at 37 °C with 5% CO_2 in the DMEM/F12 medium (1:1, Gibco; USA) supplemented with 1% GlutaMAX (Thermo Fisher Scientific; USA), penicillin/streptomycin solution with a concentration of 100 U/mL and 100 µg/mL, respectively (Gibco; USA), and 10% fetal bovine serum (Dia-M; Russia).

The cell culture of normal human keratinocytes (NHK, pool of five donors) was acquired from Perspektiva SPA LLC (Novosibirsk, Russia). The cells were cultured at 37 oC with 5% CO2 in the Keratinocyte SFM medium (Gibco; USA) containing 1% GlutaMAX (Gibco; USA), 1% antibiotic/antifungal (Gibco; USA), 50 µg/mL of BPE (Gibco; USA), 10 ng/mL of EGF (Gibco; USA).

The cells were grown in culture flasks with a surface area of 25 cm^2 or in Petri dishes with a diameter of 60 mm (Corning; USA). The medium was replaced with the fresh one every other day.

Cell cycle analysis

The cell cycle was assessed based on the Edu incorporation (Click-iT Plus EdU Alexa Fluor 488; ThermoFisher, USA) and the amount of DNA estimated by staining with Propidium Iodide (PI) (Sigma; USA). The cells were seeded into wells of the six-well plate and grown before reaching 50% confluency. Then Edu was added, and the cells were stained in accordance with the manufactirer's guidelines. After that the cells were incubated with RNase solution (100 µg/mL) and PI (1 µg/mL) for 30 min. Detection was performed using the ZE5 flow cytometer (Bio-Rad; USA) and the Everest 2.4.0.1365 software (Bio-Rad; USA).

The strongly positive Edu staining was typical for cells in S phase. The strongly positive PI staining was typical for cells with the doubled DNA content being through the G2/M phase of cell cycle.

Comparison of cell proliferation rates

The cell proliferation rates of the wild type (WT) HaCaT cells and *mutTP53* knockout (dp53) cells were compared using CytoTrace Red CMTPX (AAT Bioquest; USA). The cells were seeded into wells of the six-well plate and grown before reaching 50% confluency. Then the cells were stained with CellTracer (according to the manufacturer's guidelines) and cultured for 24 h. Detection was performed using the ZE5 flow cytometer (Bio-Rad; USA) and the Everest 2.4.0.1365 software (Bio-Rad; USA). The decrease in fluorscence intensity (dilution of the label) reflects the number of cell divisions.

Scratch test: assessment of the cell monolayer defect closure rate

To perfrom the scratch test, 50,000 of the wild type (WT) HaCaT cells, *mutTP53* knockout (dp53) HaCaT cells or normal human keratinocytes (NHK) were added to the wells of the 24-well plate and pre-incubated in the complete culture medium at 37 °C with 5% CO_2 until the monolayer was formed in the well. Then standardized damage to the monolayer was caused using the plastic scraper, the cells were washed with the DPBS solution and grown in the complete culture medium for three days. Each well was photohraphed along the entire length of the scratch every day. The images were processed using the skimage library [12]. The area not occupied by cells (defect area) was calculated 0, 24, 48, and 72 h after scratching, and the ratio compared to the baseline defect area (relative defect area) was defined. The experiment was run in triplicate.

Proteomics data

The analysis of the earlier acquired proteomics data [13] deposited in the ProteomeXchange Consortium (available from http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD030700) was carried out using the MaxQuant software (v1.6.3.4). Three biological replicates per line (wild type HaCaT cells or *mutTP53* knockout HaCaT cells) were analyzed in three technical replicates.

Estimation of gene expression

RNA was extracted using the RNeasy Kit (QIAGEN; USA) in accordance with the manufacturer's protocol. The amount of RNA obtained was measured with the NanoDrop 2000c spectrophotometer (Thermo Scientific; USA). The reaction of reverse transcription was carried out using the MMLV RT kit (Evrogen; Russia) according to the standard protocol by adding 1 µg of RNA at a time to the reaction. qPCR was performed uding qPCRmix-HS SYBR+LowROX (Evrogen; Russia). The reaction was repeated three times for every gene and sample. *GAPDH* was used as a reference gene. The primers used are provided in Table 1.

Generation of multilayered skin equivalent

To generate the organotypic model, wild type (WT) HaCaT cells, *mutTP53* knockout HaCaT cells or normal human keratinocytes (NHK) were grown in the Millipore PIHP01250 polycarbonate cell culture inserts with the pore size of 0.4 µm used in a 24-well plate. The membrane inserts were placed in the plate wells, after that 700,000 HaCaT cells (wild type or *mutTP53* knockout cells) were added to the apical compartment and 700 µL of the Submerse medium (EpiLife (Gibco; USA) containing 1% GlutaMAX (Gibco; USA), 1% antibiotic-antimycotic (Gibco; USA), 1% HKGS (Gibco; USA), 10 ng/mL of EGF (Gibco; USA), and 1.5 mmol of $CaCl_2$ (Sigma; USA)) were added to the basolateral compartment. The cells were incubated for 24 h (37 °C, 5% CO_2) to ensure cell attachment and proliferation activation.

Table 1. Primers used during the study

Gene	Primer sequences
GAPDH	Forward 5`-TCGACAGTCAGCCGCATCTTCTTT-3` Reverse 5`-ACCAAATCCGTTGACTCCGACCTT-3`
LAMB3	Forward 5`-TGGCTGAAGATGAGACCATTC-3`; Reverse 5`-GGTAGATGAAGCTCGGAGAAAC-3`
LAMC2	Forward 5`-TGGATGAGTTCAAGCGTACAC-3`; Reverse 5`-CTTTTAGCAAGATTGGCACGG-3`

After 24 h the medium was completely removed from the apical compartment, while in the basolateral compartment it was replaced with 450 µL of the growth medium at the interface of phases (ALI-medium: Submerse + 10 ng/mL of KGF (Gibco; USA) and 1 mmol of L-ascorbic acid (Sigma; USA)). The cells were grown for 10 days, and the growth medium was replaced by fresh medium every day.

Immunofluorescence staining

The cell models obtained were fixed in 4% formalin and processed by standard histology methods: dehydrated in the series of alcohols with the increasing concentrations, embedded in paraffin blocks, and sliced using microtome.

To perform immunofluorescence staining, the cells were incubated with primary antibodies against KRT5 (ab52635; Abcam, USA), CK10 (ab9025; Abcam, USA) and Alexa Fluor 488-conjugated (ab150105; Abcam, USA) or Texas Red-conjugated (ab6793; Abcam, USA) secondary antibodies. All the specimens were stained simultaneously using the same reagent kit (dilutions of antibodies and buffers).

Estimation of electrical resistance

Transepithelial electrical resistance (TEER) of the skin models generated using cells of various types was defined with the EVOM voltmeter (World Precision Instruments, Inc.; USA). For that growth medium in the basal compartment was replaced with 0.9% NaCl and 300 µL of 0.9% NaCl were added to the apical compartment of the membrane insert. After that electrodes were placed in the apical and basolateral compartments of the membrane insert, and electrical resistance (TEER) of the membrane insert with the cultured cells (skin equivalent) was measured.

Data analysis

The results obtained were processed using R programming language for statistical analysis [14]. The intergroup differences between the studied parameter values were defined using the t-test with the Benjamini-Hochberg procedure for multiple testing correction. The differences were considered significant at $p < 0.05$. The data are presented as M ± *m*.

RESULTS

Assessment of the actively proliferating cells based on the Edu (analogue of Brdu) incoporation in DNA showed that the wild type HaCaT proliferated more actively than the *mutTP53* knockout cells. In S phase there were 45.6 ± 3.2% wild type cells (HaCaT WT) on average, while the average number of *mutTP53* knockout cells (HaCaT dp53) was 34.1 ± 2.9% (Fig. 1). These data were also confirmed by the results of the CellTracer label dilution assay performed after 24 h of cell growth.

The scratch test showed that the defect closure rates of the NHK and WT HaCaT cell lines were almost equal. On day three there were 54% ± 9% and 66% ± 5% of the baseline defect areas for NHK and WT HaCaT, respectively. At the same time the *mutTP53* knockout HaCaT cells showed a significant increase in the defect closure rate: on day three there were 12% ± 3% ($p < 0.05$ compared to the corresponding group of WT HaCaT) of the baseline defect area (Fig. 2A, B).

A comparative panoramic proteome study of the wild type and *mutp53* knockout HaCaT cells was previously conducted [13]. When performing data analysis a total of 2080 proteins were identified based on two or more peptides (potential contaminants, proteins identified based on one site, and false-positives were excluded). Among the proteins identified, 27 proteins showed differences in expression between the assessed lines (FDR < 0.05). Alterations in expression of LAMC2 and LAMB3 proteins were the most interesting: the expression was significantly higher in the *mutTP53* knockout cells (4.95 and 4.58 times higher, respectively).

The data of proteomics study focused on *LAMC2* were confirmed by real-time PCR (Fig. 2): the *LAMC2* expression was significantly higher in the *mutTP53* knockout cells (9.96 ± 1.92, $p < 0.05$). However, there were no significant differences in the *LAMB3* expression between the lines assessed.

When growing 3D cultures, all three lines formed multilayered skin equivalents (Fig. 3A). However, only normal human

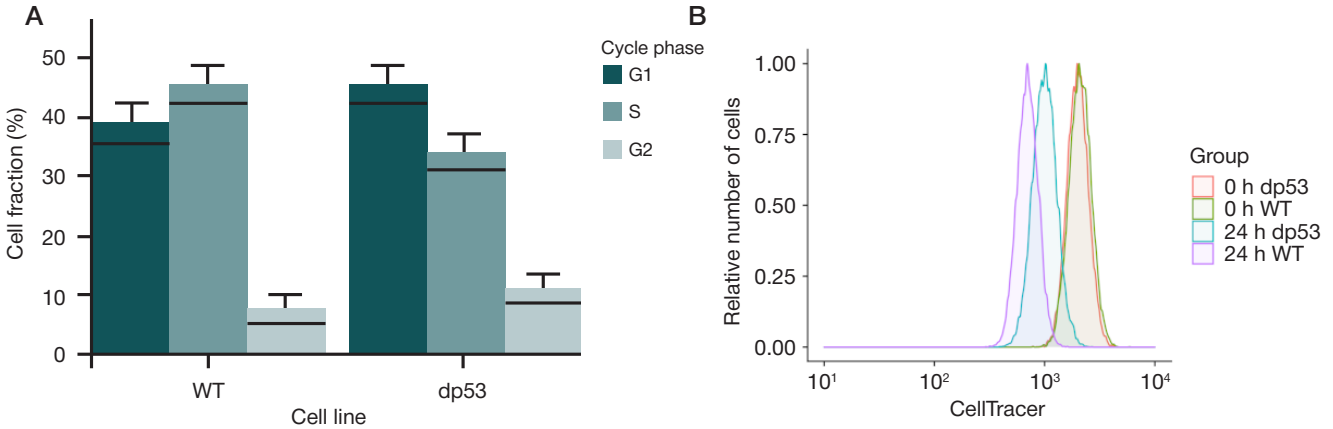

Fig. 1. Proliferative activity of HaCaT cells. **A**. Distribution of wild type (WT) HaCaT cells and *mutTP53* knockout (dp53) HaCaT cells by cell cycle phases. **B**. Proliferative activity of wild type (WT) HaCaT cells and *mutTP53* knockout (dp53) HaCaT cells

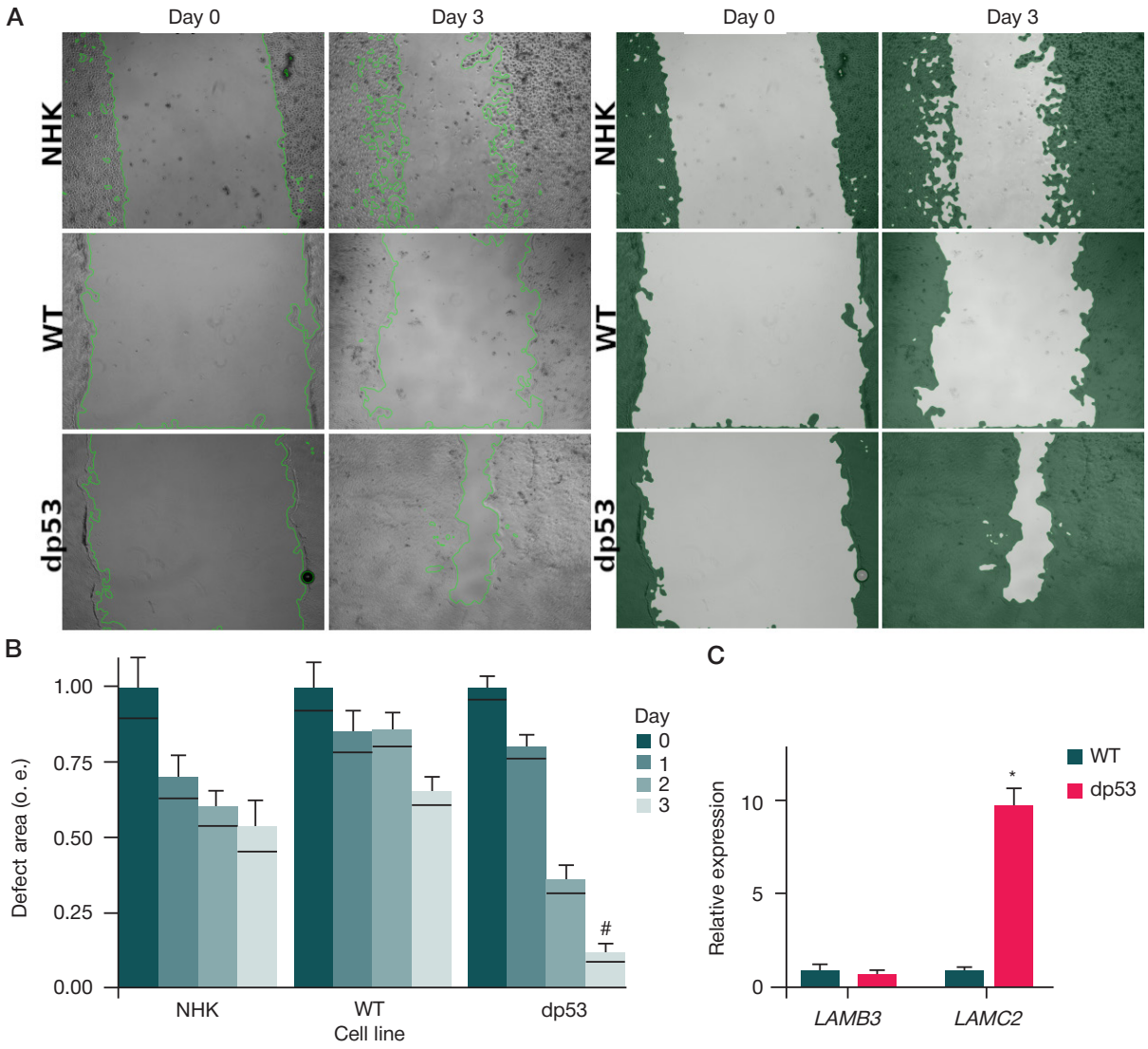

Fig. 2. Scratch test. Rates of monolayer defect closure by various cell lines: normal keratinocytes (NHK), wild type (WT) HaCaT cells, and *mutTP53* knockout (dp53) HaCaT cells. **A**. Light microscopy; magnification ×40; day 0 (immediately after injury) and day 3 after scratching. **B**. Generalized chart of the relative defect area observed on days 1, 2, 3 after scratching. **C**. Relative expression of genes *LAMB3*, *LAMC2*. * — significant differences compared to wild type HaCaT ($p < 0.05$); # — significant differences compared to NHK on the corresponding day ($p < 0.05$)

keratinocytes (NHK) showed stratification typical for normal human epidermal cells and formed the stratum corneum.

The well-defined stratum corneum was observed in the organotypic model of epidermis formed by normal human keratinocytes (NHK).

There were no clear cell stratification and stratum corneum formation in the organotypic models of epidermis formed by the wild type (WT) HaCaT keratinocytes and *mutTP53* knockout HaCaT cells. KRT5 expression was observed in all layers, and the expression levels were comparable (Fig. 3A).

When measuring transepithelial electrical resistance (TEER) in the organotypic model of epidermis formed by normal keratinocytes, electrical resistance increased throughout the observation period.

Organotypic models of epidermis formed by various cell types were different in terms of both maximum transepithelial electrical resistance values and the dynamic changes in transepithelial electrical resistance observed within five days (Fig. 3B).

The highest transepithelial electrical resistance (TEER) value was found in the model of epidermis formed by normal keratinocytes (NHK): 5712 ± 146 ohm·cm^2. In the models formed by the wild type HaCaT keratinocytes and *mutTP53* knockout HaCaT cells, the TEER values were almost five times lower: 964 ± 82 and 1088 ± 91, respectively ($p < 0.05$ compared to NHK).

Transepithelial electrical resistance increased faster between days two and four with subsequent getting to plateau in the organotypic model of epidermis formed by normal human keratinocytes. The values of this parameter in the model of epidermis formed by the wild type HaCaT keratinocytes and *mutTP53* HaCaT cells were lower compared to the model formed by NHK and got to plateau by day two.

The increase in transepithelial potential is associated with the formation of intercellular contacts and stratification of epithelium followed by the stratum corneum formation [15] that is observed in the model of epidermis formed by normal keratinocytes and is absent in the models formed by HaCaT keratinocytes, regardless of the *mutTP53* knockout (Fig. 3A).

DISCUSSION

It is known that mutant p53 in the HaCaT cells is associated with the increased cell growth and proliferation rates, which has been confirmed experimentally in the previously reported studies [5]. It is also associated with the cells' inability to normally differentiate and form the full-fledged stratum corneum.

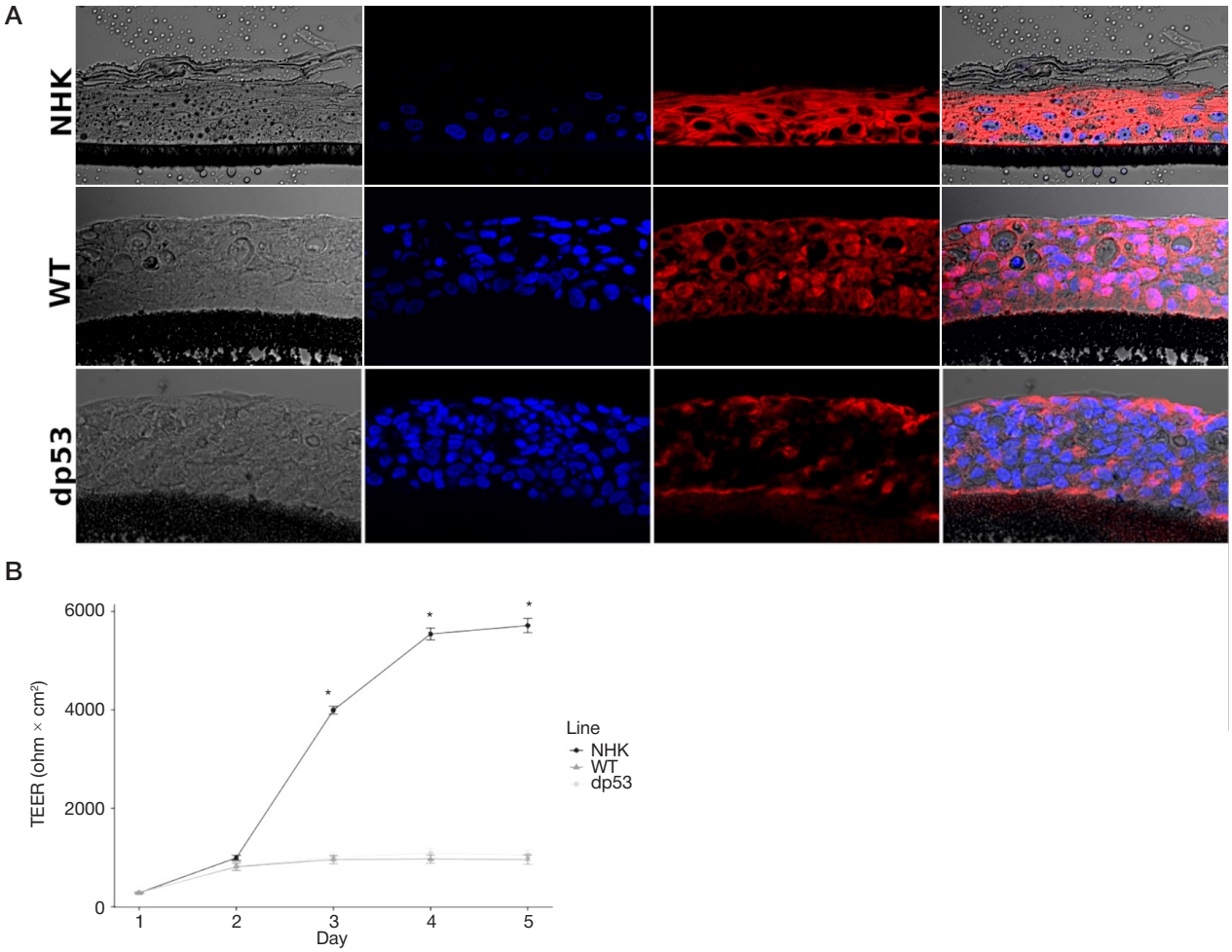

Fig. 3. Multilayered skin equivalent formation by keratinocytes of different lines: normal keratinocytes (NHK), wild type (WT) HaCaT cells, and *mutTP53* knockout (dp53) HaCaT cells. **A**. Immunofluorescence microscopy. Stain: nuclei (DAPI) — *blue*, KRT5 — *red*; magnification ×400. **B**. Transepithelial electrical resistance (TEER) values

According to the comparative proteomics study of primary human keratinocytes and HaCaT keratinocytes, one of the most noticeable differences is the expression of laminin-5 subunits, the α3, β3, and γ2 chains (LAMA3, LAMB3, LAMC2), that is significantly decreased in the HaCaT cells [16]. When discussing the findings, the authors of the above paper assume that this feature determines the abberrant nature of the HaCaT cells' differentiation program.

Laminins, being the main component of extracellular matrix, play an important role in cell adhesion, differentiation, and signal transmission [17]. The laminin-5 complex is essential for stabilization of dermoepidermal junction via binding to integrins α3β1, α6β4 and type VII collagen. Laminin-5 also plays a vital role in cell migration. It has been shown that downregulation of the gene encoding laminin-5 in keratinocytes results in reduced keratinocyte adhesion and impaired epidermal regeneration [18]. However, the increased expression of LAMB3 and LAMC2 subunits is often associated with cancer. As a result, tumor cells are characterized by higher migration rate and invasiveness [19].

Our findings show that *mutTP53* knockout in the HaCaT cells does result in the increased expression of LAMB3 and LAMC2 (the data of cell proteomics study). However, it is worth mentioning that the scratch test has revealed no significant differences in the defect closure rates between normal keratinocytes and the wild type HaCaT cells on day three after scratching, while the defect closure rate of the *mutTP53* knockout cells is significantly higher. High defect closure rate shown by the *mutTP53* knockout HaCaT cells in the scratch test should be associated with the cells' migration activity, but not with their proliferation rate, since in these cells it is significanly lower than in the wild type HaCaT cells.

Apparently, mutations in *TP53* have no significant effect on the gene's ability to regulate migration, while gene inactivation (knockout) results in the significantly increased cells' ability to migrate, including compared to normal human keratinocytes. As a result, the *mutTP53* knockout HaCaT cells acquire the features of cells with pro-carcinogenic phenotype.

It should be also noted that the elevated expression of laminin-5 resulting from the *mutTP53* knockout has shown no effect on the HaCaT keratinocytes' ability to form the full-fledged stratified epithelium in the organotypic model of epidermis. This is manifested in the lack of the stratum corneum formation and low transepithelial electrical resistance (TEER) comparable to that of the wild type cells.

Thus, our findings confirm the regulatory role of mutant p53 in the HaCaT cells in terms of laminin-5 expression, but make it impossible to associate low expression levels with abberrant differentiation program in these cells.

CONCLUSIONS

Mutant *TP53* in the HaCaT cells is a negative regulator of laminin-5 expression. The *mutTP53* gene knockout results in the increased HaCaT cells' migration activity, but does not promote normalization of differentiation program. The data obtained supplement the information about the functional features of mutant *TP53* in the HaCaT cells.

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