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Knock-Out of Receptor-Interacting Serine/Threonine Kinase 4 (RIPK4) Induces De-Differentiation Process in Keratinocytes*

Objective: Self-renewal of the skin is provided by progenitor keratinocytes that are localized to basal layer. The ones that commit to differentiate start to migrate upward to form the suprabasal layers of the epidermis. Receptor interacting serine/threonine kinase 4 (RIPK4) is is important for the growth and differentiation of the skin. The aim of this study is to analyze the effect of depletion of RIPK4 on differentiation status of keratinocytes.

Materials and Methods: RIPK4 knocked-out HaCaT (RIPK4 KO) keratinocytes were used in the experiments. Undifferentiated basal-like state and differentiated HaCaT cells were included for differentiation status comparisons. The morphology of the cells was analyzed by microscope. The levels of the RIPK4 protein were determined with western blot. To follow the growth rates, the cells were counted for 4 days. For transfection efficiency analysis, the cells were transfected with β-galactosidase expressing plasmids. The levels of differentiation-specific markers were determined by real-time-PCR.

Results Morphologically RIPK4 KO cells were different from HaCaT cells which show the tendency to grow in cell clusters. RIPK4 KO cells showed resemblance to undifferentiated basal-like state cells which were more rounded and scattered. Both RIPK4 KO and basal-like state cells had lower RIPK4 protein than HaCaT cells. In parallel with similarity to undifferentiated cell morphology, RIPK4 KO cells were more proliferative, showed higher transfection efficiency and expressed lower levels of keratinocyte differentiation markers compared to HaCaT cells.

Conclusion: The results showed that stable depletion of RIPK4 induces the de-differentiation process in keratinocytes.

Key words: *Cell differentiation; keratinocytes; gene expression; gene knock-out*

Reseptörle Etkileşen Serin/Treonin Kinaz 4 (RIPK4)'ün Keratinosıtlerde Yok Edilmesi Farklılaşma Sürecini Geriye Döndürüyor

Amaç: Derinin kendini yenilemesi bazal katmanda yerleşik öncül keratinositlerle sağlanır. Farklılaşacak olan hücreler yukarı doğru göç ederek epidermisin üst katmanlarını oluşturur. Reseptörle etkileşen serin/treonine kinaz 4 (RIPK4) derinin büyümesi ve farklılaşması için önemlidir. Bu çalışmanın amacı RIPK4'ün keratinositlerde yokluğunun HaCaT hücrelerinin farklılaşma süreci üzerindeki etkisini araştırmaktır.

Gereç ve Yöntem: Bu deneylerde RIPK4 geni işlevsizleştirilen HaCaT (RIPK4 KO) keratinositleri kullanılmıştır. RIPK4 KO'un farklılaşma statüsünü karşılaştırmak amacıyla, farklılaşmamış bazal-benzeri ve farklılaşmış HaCaT hücreleri, deneylere dâhil edilmiştir. Hücrelerin morfolojileri mikroskopla incelenmiş ve RIPK4 protein seviyeleri Western blot analizi ile belirlenmiştir. Hücrelerin büyüme hızları, hücrelerin 4 gün boyunca sayılması suretiyle takip edilmiştir. Transfeksiyon veriminin analizi için hücreler β-galaktosidaz üreten plazmidlerle transfekte edilmiştir. Farklılaşmaya özgü markörlerin gen ekspresyon seviyeleri real-time PCR ile ölçülmüştür.

Bulgular RIPK4 KO hücreleri kümeler halinde büyümeye eğilimli HaCaT hücrelerinden morfolojik olarak farklı bulunmuştur. RIPK4 KO hücrelerinin daha yuvarlak ve birbirinden ayrık büyüyen farklılaşmamış bazal-benzeri hücrelere benzediği gözlemlenmiştir. Hem RIPK4 hem de bazal-benzeri hücrelerde RIPK4 seviyelerinin düşük olduğu tespit edilmiştir. Farklılaşmamış hücre morfolojisine olan benzerliğine paralel olarak RIPK4 KO hücrelerinin HaCaT hücrelerine kıyasla daha hızlı bölündüğü, daha kolay transfekte edildiği ve farklılaşmaya özgü markörlerin ekspresyon seviyelerinin düşük olduğu belirlenmiştir.

Sonuç: Sonuçlar, keratinositlerde RIPK4'ün kalıcı olarak yok edilmesinin hücrelerin farklılaşmasını geriye döndürdüğünü göstermiştir.

Anahtar Kelimeler: *Hücre farklılaşması; keratinositler; gen ekspresyonu, gen devirme*

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Introduction

The epidermis, the outer layer of the skin, functions as a barrier that prevents water loss and protects the organism from environmental insults. To perform this barrier function, the epidermis is formed by stratified keratinocyte layers with different chemical composition and morphological characteristics (1, 2). The basal layer of the epidermis

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contains progenitors of keratinocytes that are responsible for continuous self-renewal of the skin. The ones that commit to differentiate start to migrate upward to form the suprabasal layers of the epidermis including spinous, granular and the cornified layers (3). Keratinocyte differentiation status can be determined with analysis of keratinocyte layer specific markers (2, 4). RIPK4, which is a member of RIPK (Receptor Interacting Protein Kinase) serine/threonine kinase family, is a critical regulator of keratinocyte differentiation (5). In human, RIPK4 mutations, which lead to inactivation of RIPK4's kinase activity, were associated with autosomal recessive Bartsocas-Papas syndrome, which is characterized by severe congenital anomalies of epidermal development (6, 7). Similar to human, RIPK4 deficient mice (RIPK4-/-) had serious epidermal development defects (5).

In addition to in vivo studies, the mechanism of RIPK4 function in keratinocyte differentiation was analyzed in vitro by using several cell lines including immortal HaCaT epidermal keratinocytes (8), OKF6 oral keratinocytes and primary human epidermal keratinocytes (9). In these studies, the cells were transiently transfected with RIPK4 targeting siRNAs and due to the limited stability of siRNAs inside the cells; the analyses were performed in a short period of time changing from 24 to 36 hours. So far, there is no study in the literature that stably depletes RIPK4 in keratinocyte cell lines. For studies that focus on the investigation of keratinocyte differentiation and epidermal development, HaCaT cells were preferred over other cell lines as an in vitro model due to its prominent features including their immortal status and easy maintenance in regular growth media (10). In addition HaCaT cells can allow the manipulations of the differentiation status. It has been previously demonstrated that when differentiated HaCaT cells were grown in low Ca^{+2} containing media, they can turn into cells that look like basal-layer-localized keratinocytes (basal-like state HaCaT cells), which makes these cells an appealing in vitro model to study keratinocyte differentiation (11, 12). Previously, a RIPK4 knocked-out HaCaT cells (RIPK4 KO) were established by using CRISPR/Cas9 technology in our laboratory (13). In this particular study, the effect of stable RIPK4 depletion on keratinocyte differentiation state was analysed by using previously established RIPK4 KO cells.

Materials and Methods

Cell Culture: HaCaT cells and established RIPK4 KO cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Differentiated HaCaT cells turned into basal-like state cells by growing them for at least three weeks at calcium-free DMEM medium supplemented with 10% chelexed FBS (calcium depleted), 0.032 mM calcium chloride, 1% penicillin-streptomycin, 2mM Glutamine and 1 mM sodium pyruvate as suggested in Deyrieux and Wilson (11) paper. Indicated tissue culture medium and ingredients were obtained from Thermo Fisher Scientific, USA. Chelex® 100 Chelating Resin obtained from BioRad, USA

Application of CRISPR/Cas9 System to Obtain RIPK4 KO Cells: The details of the protocol and the supplementary figure explaining the CRISPR/Cas9 approach can be found in Sumer et al., 2019 paper (13).

Transfection: For transfection of p3xFlag-RIPK4 CMV/DEST plasmid, which is previously described in Dinçer et al., 2020 (14), Lipofectamine 2000 reagent (Thermo Fisher Scientific, USA) and for transfection of RIPK4 specific si-RNA's (Thermo Fisher Scientific, USA), Lipofectamine RNAimax (Thermo Fisher Scientific, USA) reagents were used according to the manufacturer's instructions. si-RNA transfected cells were analyzed 36 h after transfection. For determination of transfection efficiency, the cells were transfected with β-galactosidase expressing plasmid (pCMV-β-Gal). 24 h after transfection, the cells were lysed with 50 mM Tris.HCl pH:7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 (TNTE) lysis buffer supplemented with protease and phosphatase inhibitors (Sigma-Aldrich, USA) and the protein concentration in the lysates was determined by using Pierce BCA protein assay kit (Thermo Fischer Scientific, USA). The β-galactosidase activity in the lysates, which contain an equal amount of protein, was determined by using o-nitrophenyl-β-d-galactopyranoside (ONPG) (Sigma-Aldrich, USA) as a substrate.

Western Blot Analysis: Cells were lysed with ice-cold TNTE lysis buffer supplemented with protease and phosphatase inhibitors (Sigma-Aldrich, USA). The protein concentration in lysates was determined by Pierce BCA protein assay kit (Thermo Fisher Scientific, USA) and 25 µg from each sample was loaded on SDS polyacrylamide gel. After gel electrophoresis, proteins were transferred to nitrocellulose membrane (Bio-Rad, USA). The membrane was blocked in 5% BSA in 0.05% Tween 20 containing Tris-buffered saline (TBS) for one hour. As primary antibodies anti-rabbit-RIPK4 (Cell Signaling Technology, USA) and anti-mouse-β-actin (Abcam, UK) and corresponding horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, USA) were applied according to manufacturer's instructions. Membranes were rinsed five times with TBS with 0.05% Tween 20 for five times between antibody applications. After the membrane was treated with ECL Plus Western blotting detection kit (Bio-Rad, USA), protein bands were visualized and quantitated by ChemiDoc MP Imaging System (Bio-Rad, USA).

Analysis of the Keratinocyte Differentiation-Specific Markers by Real-time Polymerase Chain Reaction (Real-time PCR): Total RNA from HaCaT, Basal, RIPK4 KO and si-RIPK4 transfected HaCaT cells were extracted using RNA Extraction kit (Omega Bio-tek, USA). RNA was reverse transcribed into cDNA with High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, USA). Real-time PCR was performed in triplicate for each condition by using

LightCycler®480 Instrument II (Roche Life Science, Switzerland). For 20 µL reaction 10 µL Express SYBR™ GreenER™ qPCR Supermix (Thermo Fisher Scientific, USA) was used along with 100 ng cDNA and 200 nM primers. Results were determined using the comparative Ct method ($2^{-\Delta\Delta Ct}$) with GAPDH gene as an endogenous control.

Analysis of the Growth Rate: $6x10^4$ cells were seeded into 25 cm^2 flask and growth curve was established through counting viable cells for 4 days through BD Accuri C6 flow cytometry (Becton Dickinson, USA). Doubling times were calculated by using freely available software (http://www.doubling-time.com/compute.php).

Statistical Analysis: Statistical significance values were determined either by one-way or two-way ANOVA tests for three set comparisons and P values were adjusted with Tukey's post-testing method through Graph Pad software version 8. For two set comparisons unpaired, two-tailed Students's *t-test* was applied. Error bars represent mean ± S.D. of three independent experiments, each conducted in triplicate. P<0.05 was used as the threshold for statistical significance.

Results

RIPK4 KO Shows Basal-like State Keratinocyte Morphology: Initially, the morphology of RIPK4 KO cells was examined and it was observed that RIPK4 KO cells were more rounded and scattered on the plate in contrast to cuboidal HaCaT cells that show strong cell to cell contacts and tendency to grow in cell clusters (Figure 1a). Indeed, the observed morphology of RIPK4 KO cells was similar to undifferentiated basal-like state HaCaT cells (Figure 1a). Due to the similarity in morphology, RIPK4 KO cells were investigated in terms of specific characters which were associated with basal-like state HaCaT cells. Initially, basal-like state HaCaT cells were obtained by growing HaCaT cells in low Ca^{+2} containing media for at least three weeks with regular passaging (Figure 1b). As depletion of RIPK4 leads to basal-like state phenotype, RIPK4 protein levels were evaluated among basal-like HaCaT cells that were grown in low Ca^{+2} medium, HaCaT cells and RIPK4 KO cells those were both grown in high Ca^{+2} medium and found that RIPK4 levels were significantly suppressed in RIPK4 KO cell line (5 fold) and basal-like state HaCaT cells (2 fold) compared to parental cells (Figure 1c).

RIPK4 KO has Enhanced Growth Characteristics Compared to HaCaT cells: As RIPK4 KO cells exhibited similar properties with basal-like HaCaT cells, we assessed the growth rate of RIPK4 KO cells (grown in high calcium) along with basal-like state HaCaT cells (grown in low calcium) and differentiated HaCaT cells (grown in high calcium) through monitoring growth by counting viable cells for 4 days and calculating doubling time. Basal-like HaCaT cells showed significant enhancement in cell growth compared to the differentiated HaCaT cells grown in regular high calcium medium (Figure 1d). In addition, the depletion of RIPK4 in RIPK4 KO significantly increased the cell proliferation rate compared to the parental cells. In accordance with enhanced growth rate, the doubling time of RIPK4 KO and basal-like state cells were decreased compared to parental HaCaT cells (Figure 1d).

Transfection Efficiency is Higher in RIPK4 KO Cells Compared to HaCaT Cells: It is not easy to transfect HaCaT cells in order to express gene of interest exogenously. As the transfection efficiency of basal-like state HaCaT cells was higher than HaCaT cells, the transfection efficiency of RIPK4 KO was analyzed along with basal-like state and HaCaT cells. The transfection efficiency of basal-like state cells was higher than HaCaT cells (4 fold). Besides, the transfection efficiency of RIPK4 KO cells was also enhanced compared to HaCaT cells (2.5 fold) (Figure 1e).

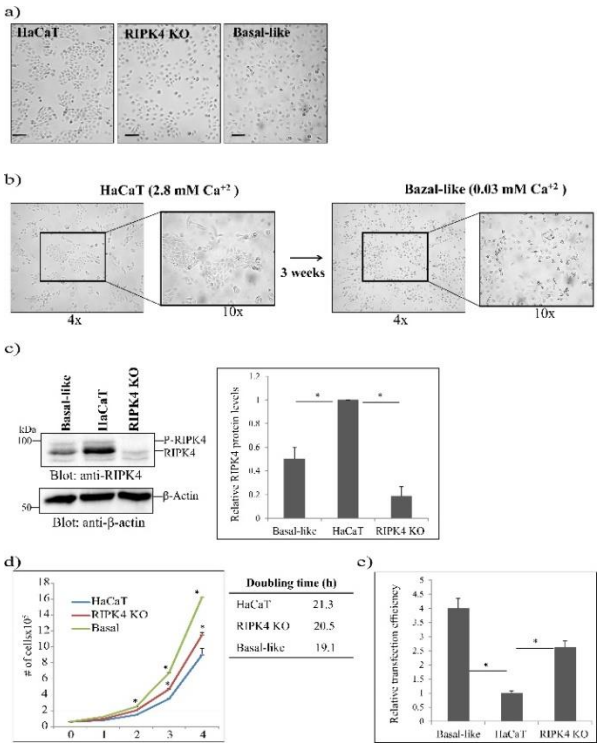

Figure 1. Analysis of RIPK4 KO cell line. RIPK4 KO clone's morphology was compared with HaCaT and basal-like HaCaT cells (a). Reversion of cuboidal HaCaT cells**,** grown in 2.8 mM Ca^{+2} medium, into dispersed basal-like HaCaT cells through culturing in 0.03 mM Ca^{+2} medium for three weeks (b). RIPK4 levels in basal-like, HaCaT and RIPK4 KO cells were analyzed by Western blot (c-left side). The graph indicated the RIPK4 levels normalized through β-actin levels (c-right side). The growth rate of HaCaT, RIPK4 KO and basal-like cells (d-left side) and corresponding doubling times (d-right side). The graph indicated the comparison of transfection efficiency between HaCaT, basal-like and RIPK4 KO cells (e). Error bars represent mean ±SD (n=3). *represents P<0.05. Scale bar represents 250 µM size.

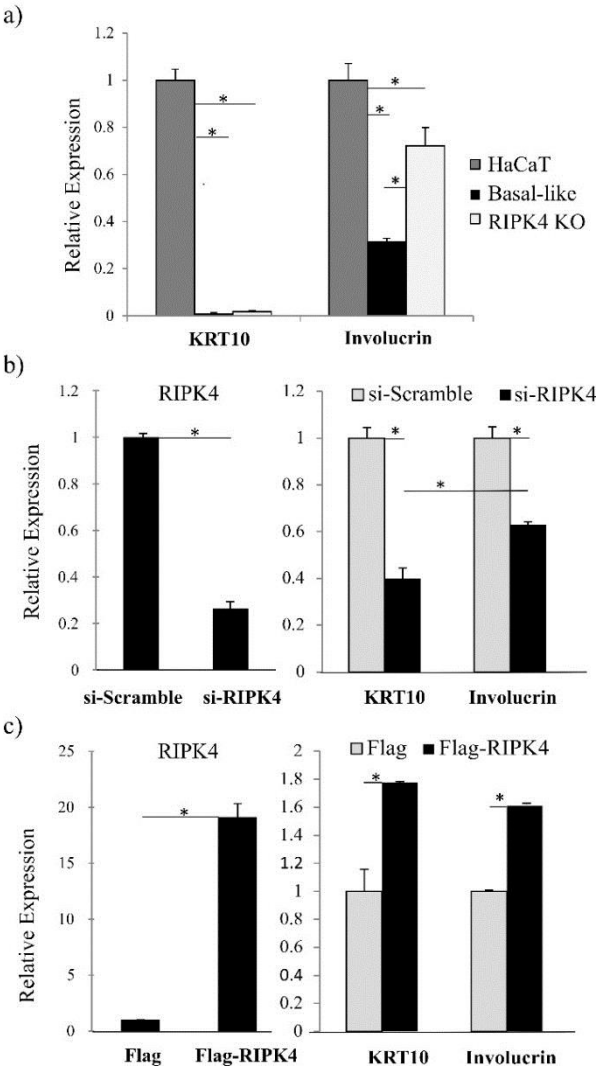

Figure 2. Analysis of differentiation markers. Relative expression levels of *KRT10* and *Involucrin* in HaCaT, basal-like and RIPK4 KO cells (a), in si-Scramble and si-RIPK4 transfected HaCaT cells (b), in Flag and Flag-RIPK4 overexpressing vectors transfected cells (c) were determined by real-time PCR. Error bars represent mean ±SD (n=3). * represents P<0.05.

Transfection Efficiency is Higher in RIPK4 KO Cells Compared to HaCaT Cells: It is not easy to transfect HaCaT cells in order to express gene of interest exogenously. As the transfection efficiency of basal-like state HaCaT cells was higher than HaCaT cells, the transfection efficiency of RIPK4 KO was analyzed along with basal-like state and HaCaT cells. The transfection efficiency of basal-like state cells was higher than HaCaT cells (4 fold). Besides, the transfection efficiency of RIPK4 KO cells was also enhanced compared to HaCaT cells (2.5 fold) (Figure 1e).

The Assessment of Keratinocyte Differentiation Markers in RIPK4 KO Cells: In order to assess the keratinocyte differentiation status of RIPK4 KO, we next analyzed the expression levels of keratin 10 (KRT10) and involucrin mRNAs, as an early and late keratinocyte differentiation-associated markers in RIPK4 KO along with HaCaT and basal-like state HaCaT cells by using real-time PCR. The *KRT10* mRNA expression level was significantly reduced in basal-like state HaCaT cells (90 fold) (Figure 2a) compared to HaCaT cells. *KRT10* expression was significantly reduced in RIPK4 KO cells compared to HaCaT cells (55 fold) as well. In addition to *KRT10*, *involucrin'*s expression levels were also reduced in both basal-like (3.1 fold) and RIPK4 KO (1.4 fold) cells compared to HaCaT cells (Figure 2a).

In order to compare the effect of stable and transient depletion of RIPK4 expression, RIPK4 was transiently down-regulated by transfecting cells with RIPK4 targeting si-RNAs (Figure 2b). In si-RIPK4 transfected cells, the morphology of the cells did not show any difference compared to HaCaT cells grown in high calcium medium (data not shown). On the other hand, the gene expression levels of both *KRT10* and *involucrin* were reduced (2.6 and 1.6 fold respectively) (Figure 2b). Concordantly, transient over-expression of RIPK4 in HaCaT cells increased the level of *KRT10* and *involucrin*; 1.8 and 1.6 folds respectively (Figure 2c) without changing the morphology of the cells (data not shown).

Discussion

RIPK4 is a critical regulator of keratinocyte differentiation whose deficiency in mice and human shows similar congenital abnormalities associated with abnormal epidermal development (5-7). So far, RIPK4's role in epidermal differentiation was studied either in keratinocytes obtained from RIPK4 (-/-) knock-out mice or in immortalized keratinocyte cell lines (8, 9). In keratinocyte cell lines based studies, RIPK4 was transiently depleted by transfecting cells with RIPK4 targeting si-RNAs which restricts the long term analyses of RIPK4 mediated events including keratinocyte differentiation. Therefore, in this study, RIPK4 KO clone in which RIPK4 expression was stably depleted using CRISPR/Cas9 method was analyzed. Interestingly, following the stable depletion of RIPK4, RIPK4 KO cells displayed scattered and rounded morphology, which was quite different from cuboidal HaCaT cells that show a tendency to grow in clusters (Figure 1a). Calcium is critical for the differentiation of basal layer localized keratinocytes (15). It is possible to revert the differentiated HaCaT cells back to the undifferentiated basal layer localized progenitor cells, which are referred to as basal-like state cells, by growing them in low calcium condition continuously. These progenitor cells can re-differentiate simply by switching on high calcium medium (11, 12). Interestingly stable depletion of RIPK4 expression in RIP4 KO cells, which was fivefold compared to parental cell line, shifted these cells into a basal-like state, even though they were grown in regular calcium-rich medium (Figures 1 and 2). As previously mentioned in Sumer et al., 2019 (13), RIPK4 KO

carries compound heterozygote RIPK4 mutations. One of the mutations is one nucleotide insertion that resulted in a premature stop codon and the other one is 12 nucleotides deletion. Even though 12 nucleotide deletions still lead to the expression of protein, the 5 fold suppression of RIPK4 expression compared to parental cell line indicated that deletions induced destabilization of the protein (Figure 1c).

Considering the morphological resemblance to basal-like state cells, we compared RIPK4 KO with basal-like state cells along with HaCaT cells in terms of distinctive features associated with basal-like state cells such as enhanced growth rate, increased transfection efficiency and reduced level of keratinocyte differentiation markers (11, 12). Similar to basal-like state cells, RIPK4 KO cells showed enhanced growth rate (Figure 1d) and transfection efficiency (Figure 1e) compared to HaCaT cells. Previously, it has been demonstrated that protein levels of KRT10 and involucrin decreased in basal-like state HaCaT cells compared to HaCaT cells and the fold reduction was more prominent in KRT10 than involucrin (11). In accordance with this report, both KRT10 and involucrin expression were reduced in RIPK4 KO cells and it was more apparent for KRT10 (Figure 2a). In parallel with the morphological resemblance, the depletion of RIPK4 shifted HaCaT cells more toward basal-like state cells in every aspect. However, in RIPK4 KO cells the extent of differences compared to parental cell lines was not as high as basal-like state cells (Figures 1 and 2). Indeed continuous depletion of RIPK4 in HaCaT cells turned these cells into an intermediate state between undifferentiated basal-like and differentiated HaCaT cells.

In the epidermis of RIPK4-/- mice, due to the differentiation-induced growth arrest delay at suprabasal localized cells, stratified layers organization and associated layer-specific keratinocytes markers expression are disrupted (5, 16). In accordance with, the primary keratinocytes obtained from RIPK4-/- mice's skin is unable to differentiate upon stimulation with epidermal differentiation factors such as calcium and vitamin D (16). Similar to keratinocytes obtained from RIPK4-/- mice, the depletion of RIPK4 expression strongly inhibited *KRT10* and *involucrin* expression levels in RIPK4 KO and si-RIPK4 transfected cells even though they were grown in high Ca^{+2} medium (Figures 2a and b). Moreover, basal-like state cells showed reduced RIPK4 expression compared to HaCaT cell as well (Figure 1c). These results indicated that RIPK4 is one of the important determinants for de-differentiation process which possibly requires additional Ca^{+2}-mediated factors for the full transition.

When RIPK4 was transiently depleted by RIPK4 targeting si-RNAs in HaCaT cells there was no obvious difference in terms of morphology (data not shown) indicating the long term depletion is required for the appearance of this phenotype. This situation can also be explained with the level of *KRT10* suppression, which is quite high in basal-like state and RIPK4 KO cells (90 fold and 55 fold respectively) compared to si-RIPK4 transfected HaCaT cells (2.5 fold). KRT10 is an early keratinocyte differentiation marker and a critical determinant of basal to suprabasal layer transition (17). Interestingly, RIPK4 depletion shows its effect profoundly on the KRT10 level, which emphasizes RIPK4's role in the early differentiation process. In accordance with, in our previous study, it was shown that RIPK4 interacts with basal layer-specific keratin14 (KRT14) which localizes just below KRT10 expressing spinous layer (13). In addition to the levels of differentiation markers, RIPK4 KO exhibited this intermediate phenotype between basal-like state cells and HaCaT cells in terms of transfection efficiency and growth rate as well (Figures 1d and e).

As a conclusion, within this study, a RIPK4 knocked-out clone, which exhibit an intermediate differentiation state between basal-like state and HaCaT cells, was characterized. Indeed, this particular characteristic makes this clone an appealing in vitro model to understand the molecular basis of basal to suprabasal layer differentiation. Besides, with this study the previous in vivo mice based studies that show the correlation between RIPK4 depletion and suppression of epidermal differentiation (16, 18) were confirmed in HaCaT cells. Moreover, in accordance with hypothesis, continuous depletion of RIPK4 was shown to be necessary to observe the distinct effect of RIPK4 on keratinocyte differentiation.

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