The protein deacetylase SIRT3 prevents oxidative stress-induced keratinocyte differentiation*

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*Running title: SIRT3 regulation of keratinocyte differentiation

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Keywords: keratinocyte; differentiation; ROS; SIRT3

Background: SIRT3 plays a major role in protecting against mitochondrial oxidative stress.

Results: Oxidative stress increases during keratinocyte differentiation, and SIRT3 can decrease differentiation by attenuating oxidative stress.

Conclusion: SIRT3-induced down regulation of mitochondrial oxidative stress attenuates keratinocyte differentiation.

Significance: Understanding the regulation of keratinocyte differentiation is crucial for developing new therapies against dysregulated skin differentiation and aging.

SUMMARY
Keratinocyte differentiation is a key process in the formation and maintenance of the protective skin barrier. Dysregulation in the balance of reactive oxygen species (ROS) homeostasis may play a role in keratinocyte differentiation. We have identified the mitochondrial deacetylase, SIRT3, as a key regulator of mitochondrial ROS in keratinocytes. Our studies demonstrate that SIRT3 expression is down regulated during keratinocyte differentiation, consistent with an increase in mitochondrial superoxide levels. Importantly, loss of SIRT3 expression in keratinocytes increases superoxide levels and promotes the expression of differentiation markers, while overexpression decreases superoxide levels and reduces the expression of differentiation markers. These findings identify a new role for SIRT3 in the suppression of epidermal differentiation via lowering oxidative stress.

INTRODUCTION
The epidermis functions as a protective barrier, which is comprised of four morphologically distinguished layers: basal layer, spinous layer, granular layer and cornified layer. Keratinocyte differentiation is an essential process in the formation and maintenance of the protective skin barrier. Proliferating keratinocytes in the basal layer function to regenerate the epidermis and maintain attachment to the basal lamina. Maturing keratinocytes detach from the basal layer and start the differentiation process in the spinous layer by expressing early differentiation markers, such as keratin-1 (KRT1), keratin-10 (KRT10), involucrin (INV) and transglutaminase (TGM). In the granular layer keratinocytes produce cornified envelope proteins, such as the late differentiation markers loricrin (LOR) and filaggrin (FLG). Finally, in the cornified layer, proteins are crosslinked and lipids are extruded to form the epidermal barrier (1-3).

Mitochondrial function plays an important role during keratinocyte differentiation. For example, mitochondrial membrane potential declines during differentiation and eventually mitochondrial cell death pathways are activated to induce terminal differentiation (4). Recent studies reported that induction of oxidative stress up regulates the expression of differentiation markers in keratinocytes, triggers apoptosis or terminal differentiation (5), while the loss of the antioxidant enzyme superoxide dismutase 2 (SOD2) induces senescence and differentiation in keratinocytes (6). Keratinocytes lacking the mitochondrial transcription factor A (TFAM) cannot generate
mitochondrial ROS and thus show impaired differentiation (7). However, few details are known about the molecular mechanisms responsible for controlling the levels of oxidative stress during normal differentiation.

The mitochondrial sirtuin deacetylase SIRT3 plays a critical role in the generation and clearance of mitochondrial reactive oxygen species (ROS) (8). It binds and deacetylates several mitochondrial proteins that promote mitochondrial oxidative metabolism, such as electron transport chain subunits and fatty acid oxidation enzymes (9,10). SIRT3 also dampens mitochondrial oxidative stress via deacetylation and subsequent activation of SOD2 and isocitrate dehydrogenase (IDH) (8,11-18).

As SIRT3 plays a pivotal role in ROS clearance, we sought to investigate the role of SIRT3 in regulating ROS and differentiation in keratinocytes. We show for the first time that during keratinocyte differentiation SIRT3 expression is decreased and that the levels of SIRT3 substrate NAD+ are reduced, while ROS are increased. We further demonstrate that a reduction of oxidative stress by the SOD mimetic MnTBAP or the antioxidant N-Acetylcysteine (NAC) attenuates keratinocyte differentiation. In keratinocytes with reduced SIRT3 activity, we show an increase in oxidative stress and an increase in expression of differentiation markers. We observe the opposite phenotypes in keratinocytes that overexpress SIRT3. Our findings identify a novel molecular mechanism controlling keratinocyte differentiation via ROS accumulation. SIRT3 lowers mitochondrial oxidative stress to decrease the rate of keratinocyte differentiation, while loss of SIRT3 induces keratinocyte differentiation due to aberrant ROS.

EXPERIMENTAL PROCEDURES

Cell culture - The primary normal human epidermal keratinocytes strain N and the TERT-immortalized human epidermal keratinocyte line N/TERT-1 (19) were provided by J. Rheinwald and the Cell Culture Core of the Harvard Skin Disease Research Center, Boston, MA. The primary keratinocytes and TERT-immortalized keratinocytes were cultured in K-SFM (GIBCO) supplemented with 25 µg/ml bovine pituitary extract, 100 ng/ml epidermal growth factor, 0.1 mM CaCl2 (Sigma) and 100 units/ml penicillin and streptomycin (GIBCO). Cultures were maintained at low confluence to prevent differentiation. Density-dependent differentiation was induced by plating cells at approximately 1x10³/cm² in high-density medium consisting of K-SFM:DMEM/F12 (1:1) supplemented with 25 µg/ml bovine pituitary extract, GlutaMAX™-I (GIBCO) and 100 units/ml penicillin and streptomycin. Calcium-dependent differentiation was induced by adding 1.2 mM CaCl2 to the culture medium of sub-confluent cultures.

Retroviral Transduction - Retroviral particles were produced by co-transfection of pBabe-puro plasmid control or SIRT3-Flag containing pBabe-puro plasmid with gag/pol and VSV-G packaging vectors, into HEK293T cells using FuGENE® 6 Transfection Reagent (Roche). Retroviral supernatant was harvested 36 and 60 hours post transfection, and filtered through a 0.45 µm filter. N/TERT cells were transduced with viral medium for 24 hours. Selection was performed in the presence of puromycin (2.5 µg/ml) for 1 week.

Lentiviral Transduction - Lentiviral particles were produced by co-transfection of empty or SIRT3-shRNA containing pLKO.1-puro vector with pCMV-dR8.2 and pCMV-VSVG packaging plasmids into HEK293T cells with FuGENE® 6 Transfection Reagent (Roche). Lentiviral supernatant was harvested 36 and 60 hours posttransfection, and filtered through a 0.45 µm filter. N/TERT-1 cells were transduced with viral medium and incubated for 24 hours. Selection was performed in the presence of puromycin (2.5 µg/ml) for 1 week.

Quantitative RT-PCR - Total RNA from cells was harvested using TRIzol® Reagent (Invitrogen). Total RNA was purified using the RNaseasy kit (Qiagen). RNA purity and concentration were determined using a Nano Drop spectrophotometer. cDNA was synthesized from 1µg RNA using the iScript™ cDNA Synthesis Kit (Bio-Rad). cDNA was used at a dilution of 1:250 per reaction with SYBR® Green Master Mix on Roche LightCycler® 480 Real-Time PCR System. Serial dilutions of pooled samples were used to generate a standard curve and analyzed for each gene tested. Absolute RNA levels for each gene were calculated according to the standard curve and further normalized to housekeeping genes and control.
Samples were immediately subjected to two freeze/thaw cycles on dry ice. The supernatant of the cells in NADH/NAD⁺ extraction buffer was split into two sets, one of which was used to measure the total NADH plus NAD⁺ (total NADH) content, and the other set was used to carry out the thermal decomposition of NAD⁺ to measure only the NADH content of the cell. The cycling reaction was initiated in a 96-well plate in both sets and a set of NADH standard dilutions and stopped after 1 hour. The absorbance at 450nm was determined and the NAD⁺ content could be calculated as NAD⁺ = total NADH – NADH and were normalized to cellular protein content.

**Immunofluorescence** - Cells were grown on round cover glass and treated as indicated in the text. They were fixed in 4% paraformaldehyde for 15 minutes at 4°C, permeabilized in 0.3% Triton X-100 for 15 minutes at room temperature, blocked with 5% normal goat serum for 30 minutes at 37 °C and then stained overnight at 4°C with the indicated antibodies. For loricrin immunostaining the polyclonal antibody from Covance was used (dilution 1:100). All slides were washed three times in PBS and incubated with Alexa488-conjugated rabbit secondary antibodies for 1 hour at room temperature. Nuclei were visualized by 4′,6-diamidino-2-phenylindole (DAPI) staining. Samples were rinsed, mounted on coverslips using ProLong® Gold antifade reagent (Invitrogen), and examined by fluorescence microscopy. All images were collected with a Nikon 80i upright microscope equipped with either a Plan Fluor 40X 1.3 NA objective lens or a Plan Apo 100X 1.4 NA objective lens and with epi-fluorescence illumination. Loricrin fluorescence was excited with a 480/40 filter (Chroma) and emission collected with a 535/50 filter (Chroma). Images were acquired with a Hamamatsu C8484-03 monochrome CCD camera controlled with MetaMorph 7 software. Contrast and brightness were adjusted on displayed images (identically for compared image sets) using MetaMorph 7 software and Photoshop.

**Statistical Analysis** - All values are expressed as mean ± SEM. Significant differences between two groups were evaluated by an unpaired Student t test.
RESULTS

SIRT3 expression and activity are down regulated during keratinocyte differentiation

To examine changes in SIRT3 activity during normal keratinocyte differentiation, we used the normal human epidermal keratinocyte line N/TERT-1, an hTERT-immortalized human keratinocyte cell line, which retains the ability to differentiate in vivo and in vitro (19). We examined the expression of SIRT3 during keratinocyte culture with two established stimuli that induce commitment to terminal differentiation: culture confluence (Fig. 1A - C), and elevation of extracellular calcium concentration (Fig. 1D - F) (20). Under these conditions SIRT3 expression decreased in N/TERT keratinocytes. Additionally, cellular levels of NAD(H), in particular in its oxidized state as NAD⁺, a co-substrate for sirtuin catalysis (21-23), decreased during keratinocyte differentiation while levels of NADH did not change significantly (Fig. 2A, B, C). Taken together, these findings support the model that SIRT3 expression and activity decrease during keratinocyte differentiation.

Mitochondrial and cellular superoxide levels increase during keratinocyte differentiation

As SIRT3 diminishes mitochondrial oxidative stress (8), we reasoned that a reduction in SIRT3 activity during keratinocyte differentiation may lead to increased mitochondrial ROS. Thus, we investigated alterations in levels of mitochondrial superoxide during differentiation in N/TERT keratinocytes. Notably, we detected an increase in mitochondrial superoxide levels during differentiation in both the primary normal human epidermal keratinocytes strain N, as well as the hTERT-immortalized human keratinocytes N/TERT-1 (Fig. 2D, E). We further detected increased cellular superoxide levels during differentiation (Fig. 2F).

Loss of SIRT3 affects keratinocyte morphology and growth

To directly probe the hypothesis that SIRT3 loss diminishes ROS in order to promote keratinocyte differentiation, we generated a series of stable N/TERT-1 cell lines in which SIRT3 levels were overexpressed or reduced. Using a pBabe-SIRT3 retroviral vector, we generated a stable cell line, which overexpresses SIRT3 as confirmed by quantitative RT-PCR and western blot (Fig. 3A, C). Additionally, using five different shRNA sequences targeting SIRT3 mRNA, we identified two shRNAs (shSIRT3a and shSIRT3b) that achieved efficient silencing as demonstrated by both quantitative RT-PCR and western blot (Fig. 3B, D). The growth rate and morphology of the N/TERT-1 cells overexpressing SIRT3 were comparable to the control cell line (Fig. 3E, G). In the case of the SIRT3-knockdown keratinocytes, loss of SIRT3 led to decreased proliferation (Fig. 3F) possibly shifting cells towards a differentiated morphology (Fig. 3H). The cell line shSIRT3b, with the most efficient silencing of SIRT3, stopped proliferating after 4-5 passages. For this reason the shSIRT3a line was used for subsequent experiments, and is further labeled as shSIRT3.

SIRT3 modulates mitochondrial superoxide levels in keratinocytes

SIRT3 acts through several targets to both reduce superoxide production and enhance the oxidative stress response and detoxification mechanisms (8,11-17). Consistent with the repression of mitochondrial oxidative stress by SIRT3, mitochondrial superoxide levels decreased in SIRT3-overexpressing keratinocytes (Fig. 4A) and increased with SIRT3 knockdown (Fig. 4B, C). In order to test whether this increase in mitochondrial superoxide was due to reduced detoxification or enhanced ROS production, we tested the effect of two antioxidants, SOD-mimetic manganese 5,10,15,20-tetrakis (4-benzoic acid) porphyrin (MnTBAP) or NAC, as well as the effect of the oxidant hydrogen peroxide (H₂O₂) on SIRT3-deficient keratinocytes. Treatment with MnTBAP or NAC rescued SIRT3-deficient keratinocytes by boosting superoxide detoxification. These treatments diminished the increased superoxide levels that were produced due to SIRT3 silencing (Fig. 4D, E). Furthermore, treatment with H₂O₂ elevated superoxide levels in SIRT3-deficient keratinocytes compared with similarly treated control keratinocytes, suggesting that SIRT3 deficiency also promotes the accumulation of superoxide in keratinocytes (Fig. 4F) while SIRT3 overexpression protects from H₂O₂ induced superoxide (Fig. 4G). These findings support a role for SIRT3 in reducing mitochondrial ROS by affecting both ROS production and detoxification.
Recent studies showed that mitochondrial superoxide accumulates due to dysfunction or loss of antioxidant mechanisms in keratinocytes (6,21). Furthermore, modulation of mitochondrial function can increase ROS and lead to keratinocyte senescence and increased terminal differentiation (5). Treatment with antioxidants, such as MnTBAP and NAC, has been shown to have beneficial effects on the phenotypes caused by deficient antioxidant mechanisms (22,23). In our model, incubation with MnTBAP decreased superoxide levels in a dose-dependent manner (data not shown). To test whether pharmacological detoxification of mitochondrial superoxide affects the process of keratinocyte differentiation, we treated N/TERT-1 keratinocytes with various doses of MnTBAP over 5 days of confluence-induced differentiation. As expected, markers of differentiation, such as loricrin and filaggrin, increased significantly with time, however gene expression and protein level of the differentiation marker loricrin decreased with increasing doses of MnTBAP (Fig. 5A). In keratinocytes incubated with 5 μM MnTBAP during 5 days of confluence-induced differentiation, both loricrin and filaggrin expression were attenuated by about 50% (Fig. 5B). Importantly, treatment with 2 mM NAC also showed a similar attenuation of differentiation (Fig. 5C). Even in subconfluent proliferating keratinocyte cultures where loricrin protein expression is low, incubation with 5 μM MnTBAP over 24 hours lowered loricrin levels as detected by immunofluorescence (Fig. 5D). Thus, superoxide levels are an important factor in the induction of keratinocyte differentiation.

**SIRT3 modulates keratinocyte differentiation**

Next, we examined whether the SIRT3-mediated decrease in oxidative stress directly affects the differentiation characteristics of keratinocytes. Importantly, SIRT3-overexpression decreased the appearance of late differentiation markers loricrin and filaggrin, as well as early differentiation markers, including transglutaminase, involucrin, keratin-1 and keratin-10 proteins, whereas SIRT3 knockdown increased expression of these markers (Fig. 6A - D). To investigate whether SIRT3 regulates differentiation via a mechanism of ROS, we performed similar studies in the presence of MnTBAP. Strikingly, incubation with MnTBAP over 3 days of confluence-induced differentiation attenuated the increased expression of loricrin and filaggrin in SIRT3-knockdown keratinocytes (Fig. 6E - F), and also reduced loricrin protein expression in subconfluent SIRT3-knockdown keratinocytes (Fig. 6G). Diminished SIRT3 expression drove increased oxidative stress, stimulating the expression of keratinocyte differentiation (Fig. 6H). Taken together, our data reveal a novel mechanism by which reduced SIRT3 activity contributes to elevated ROS and promotes keratinocyte differentiation.

**DISCUSSION**

This study reveals important and novel mechanistic insights into how oxidative stress levels change during normal keratinocyte differentiation. First, we showed that SIRT3 expression and levels of SIRT3 co-substrate NAD⁺ are down regulated during keratinocyte differentiation. Decreased availability of the sirtuin co-substrate NAD⁺ may lead to decreased SIRT3 activity in keratinocytes during differentiation. Given the well-established role of SIRT3 as a regulator of mitochondrial oxidative stress (8,11,18), mitochondrial superoxide levels increased in both primary and immortalized keratinocytes with differentiation. We identified SIRT3 as a key regulator of this phenotype; constitutive SIRT3 deficiency resulted in increased mitochondrial superoxide levels, while constitutive SIRT3 expression reduced mitochondrial ROS.

The mitochondrial sirtuin SIRT3 is uniquely poised to modulate both the generation of ROS and the detoxification of harmful free radicals, protecting cells from mitochondrial oxidative stress (8,11,17,18). Loss of SIRT3 triggers ROS and oxidative damage, affecting distinct cellular processes and promoting phenotypes of aging (13,14). Our data define a key role for SIRT3 in keratinocyte differentiation through regulation of ROS. Increased SIRT3 activity prevents expression of differentiation markers, and loss of SIRT3 induces keratinocyte differentiation. Importantly, addition of the antioxidant MnTBAP counteracts differentiation in SIRT3-knockdown keratinocytes, suggesting that the loss of SIRT3 induces differentiation through increased ROS. This conclusion is supported by studies showing that oxidative stress induces keratinocyte differentiation and senescence (4,6,21). For
example, defects in antioxidant capacity, such as an SOD deficiency, may lead to keratinocyte senescence and decreased thickness of the epidermis (6). It remains unclear how mitochondrial oxidative stress signaling induces and modulates keratinocyte differentiation. Previous studies suggest that ROS signaling might induce keratinocyte differentiation through the PKC/AP-1 pathway (24,25). Interestingly, SIRT3 gene expression might in turn also be regulated through AP-1 transcription factors (26) suggesting a possible regulatory pathway. Elucidating the mechanisms involved in normal epidermal keratinocyte differentiation will be crucial for understanding a number of skin diseases with abnormal epidermal keratinocyte differentiation, as well as aging. Our studies highlight keratinocyte SIRT3 as a new target that could potentially be developed to regulate disorders resulting from abnormal oxidative stress and epidermal differentiation.
REFERENCES


ACKNOWLEDGEMENTS
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ABBREVIATIONS
FLG filaggrin, hTERT human telomerase reverse transcriptase, INV involucrin, KRT1 keratin 1, KRT10 keratin 10, LOR loricrin, ROS reactive oxygen species, SIRT3 Sir2uin-3, SOD superoxide dismutase, TGM transglutaminase

TABLE 1. Sequences of primers used for qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Primers</th>
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<tr>
<td>Ribosomal protein P0 (RPLP0)</td>
<td>housekeeping gene</td>
<td>F ATCAACGGGTACAAACGAGTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R CAGATGGATCACGCAAGAAGG</td>
</tr>
<tr>
<td>Sirtuin-3 (SIRT3)</td>
<td>protein deacetylase</td>
<td>F GGGCTTGAGAGAGTGTCGGGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R TCACAGCGGGTCAGGACACC</td>
</tr>
<tr>
<td>Loricrin (LOR)</td>
<td>late differentiation marker</td>
<td>F TCATGATGCTACCCGAGGTGGTGTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R CAGAATCATGACCTGACAGACAGAAG</td>
</tr>
<tr>
<td>Filaggrin (FLG)</td>
<td>late differentiation marker</td>
<td>F GGGCACTGAAAGGCCAAAAGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R CACCATAATGAATGACTGACAGAAG</td>
</tr>
<tr>
<td>Transglutaminase (TGM)</td>
<td>early differentiation marker</td>
<td>F ATCCTCATGGTCCACGTACACA</td>
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<tr>
<td></td>
<td></td>
<td>R CCCCGAATGACTGAAT</td>
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<tr>
<td>Involucrin (INV)</td>
<td>early differentiation marker</td>
<td>F GGGTGTTTATTTATGTTGAGGTG</td>
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<tr>
<td></td>
<td></td>
<td>R GCCAGTTCCAAGAATCAAC</td>
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<tr>
<td>Keratin-1 (KRT1)</td>
<td>early differentiation marker</td>
<td>F ATTTCTGAGCTGAAATGTGACGTGTC</td>
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<td></td>
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<td>R CTTGGCATCCTGAGGGGCATT</td>
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<td>Keratin-10 (KRT10)</td>
<td>early differentiation marker</td>
<td>F TGATGGAATGAGAAATGAAATGAAT</td>
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<tr>
<td></td>
<td></td>
<td>R GTAGCAGTCTGCTCTTCTTCTTTCA</td>
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FIGURE LEGENDS

FIGURE 1. SIRT3 is differentially expressed during keratinocyte differentiation. RT-PCR analysis of N/TERT-1 cultured over 5 days after reaching confluence shows expression of SIRT3 and the differentiation markers loricrin and filaggrin during confluence-induced differentiation, A, B, and C, and during calcium-induced differentiation with addition of 1.2 mM CaCl₂. D, E, and F. Data are representative of three independent experiments. Data shown are from three biological replicates, error bars indicate SEM.

FIGURE 2. NAD⁺ levels decrease and mitochondrial superoxide levels increase during keratinocyte differentiation. A, Relative total NAD(H) levels, B, NAD⁺, and C, NADH measurements in N/TERT-1 at days 1 and 5 after reaching confluence. D and E, analysis of MitoSOX fluorescence by flow cytometry of primary keratinocytes and N/TERT-1 at days 1, 3 and 5 after reaching confluence, and F, analysis of dihydroethidium (DHE) fluorescence by flow cytometry of N/TERT-1 after days 1 and 3 after reaching confluence. Data are representative of two independent experiments. *, P <0.05; **, P < 0.01; ***, P < 0.001; n = 3.


FIGURE 4. Effect of SIRT3 activity on superoxide levels in keratinocytes. Flow cytometry analysis of MitoSOX and DHE fluorescence in control (black bars) and A, SIRT3-overexpressing (blue bars) or B and C, SIRT3-knockdown (red bars) N/TERT-1, and after overnight incubation with D, 100 µM MnTBAP, E, 5 mM NAC, or F, 100 µM H₂O₂, in control or SIRT3-knockdown cells, or G, SIRT3 overexpressing cells. Data are representative of three independent experiments. Error bars indicate SEM; *, P <0.05; **, P < 0.01; n = 3.

FIGURE 5. Effect of MnTBAP on differentiation in keratinocytes. A, Western Blot of loricrin in N/TERT-1 incubated with MnTBAP over 5 days after reaching confluence. B, RT-PCR analysis of the differentiation markers loricrin (LOR) and filaggrin (FLG) in N/TERT-1 cultured with 5 µM MnTBAP over 5 days, or C, 2 mM NAC over 3 days after reaching confluence. D, Immunofluorescent staining for loricrin (green) of N/TERT-1 treated with 5 µM MnTBAP for 24 hours before reaching confluence. Nuclei were stained with DAPI (blue). Data are representative of two independent experiments. Error bars indicate SEM; *, P <0.05; **, P < 0.01; ***, P < 0.001; n = 3.

FIGURE 6. Effect of SIRT3 activity on differentiation in keratinocytes. RT-PCR analysis of A, the late differentiation markers loricrin (LOR) and filaggrin (FLG), or B, the early differentiation markers transglutaminase (TGM), Involvedin (INV), Keratin-1 (KRT1) and Keratin-10 (KRT10) in control and SIRT3 overexpressing N/TERT-1 cultured over 5 days after reaching confluence. RT-PCR analysis of C, the late differentiation markers loricrin (LOR) and filaggrin (FLG), or D, the early differentiation markers transglutaminase (TGM), Involvedin (INV), Keratin-1 (KRT1) and Keratin-10 (KRT10) in control and SIRT3 knockdown N/TERT-1 cultured for 3 days after reaching confluence. qRT-PCR analysis of control or SIRT3 knockdown N/TERT-1 cultured with 5 µM MnTBAP over 3 days after reaching confluence, shows expression of the late differentiation markers E, loricrin, and F, filaggrin. G, Immunofluorescence staining for loricrin (green) of control or SIRT3-knockdown N/TERT-1, treated with 5 µM MnTBAP for 24 hours before reaching confluence, nuclei were stained with DAPI (blue). Data are representative of two or three independent experiments. Error bars indicate SEM; *, P <0.05; **, P < 0.01; ***, P < 0.001; n = 3. H, Schematic of changes in SIRT3 and ROS levels during keratinocyte differentiation.
Figure 1.
Figure 2.

A-D. Primary keratinocytes

E, F. N/TERT-1

Day 1 vs. Day 5 comparison.
Figure 3.

(A) Relative expression of SIRT3 and Tubulin in Ctrl and SIRT3 groups.

(B) Relative expression of SIRT3a and SIRT3b in Ctrl and SIRT3 groups.

(C) Western blot analysis of SIRT3 and Tubulin in Ctrl and SIRT3 groups.

(D) Western blot analysis of SIRT3 and Tubulin in Ctrl, SIRT3a, and SIRT3b groups.

(E) Cell count over time in Ctrl and SIRT3 groups.

(F) Cell count over time in Ctrl and shSIRT3 groups.

(G) Optical microscopy images of Ctrl and SIRT3 groups.

(H) Optical microscopy images of Ctrl, shSIRT3a, and shSIRT3b groups.
Figure 4.

A. MitoSOX Mean Fluorescence (Ex/Em 488/589) Ctrl vs SIRT3

B. MitoSOX Mean Fluorescence (Ex/Em 488/589) Ctrl vs shSIRT3

C. DHE Mean Fluorescence (Ex/Em 488/589) Ctrl vs shSIRT3

D. MitoSOX Mean Fluorescence (Ex/Em 488/589) MnTBAP (μM) 0 vs 100

E. MitoSOX Mean Fluorescence (Ex/Em 488/589) NAC (mM) 0 vs 5

F. MitoSOX Mean Fluorescence (Ex/Em 488/589) H₂O₂ (μM) 0 vs 100

G. MitoSOX Mean Fluorescence (Ex/Em 488/589) H₂O₂ (μM) 0 vs 100
Figure 5.

A

MnTBAP (μM) 0 0.625 1.25 2.5 5 10

Loricrin

Tubulin

B

Ctrl MnTBAP

* **

C

Ctrl NAC

* **

D

DAPI Loricrin Merged

Ctrl MnTBAP
Figure 6.

A-B: Relative expression of LOR and FLG in Ctrl and SIRT3 groups.

C-D: Relative expression of TGM, INV, KRT1, and KRT10 in Ctrl and shSIRT3 groups.

E-F: Relative expression of Ctrl, shSIRT3, and shSIRT3 + MnTBAP.

G: DAPI, Loricrin, and Merged images for Ctrl and shSIRT3 with and without MnTBAP.

H: Diagram showing differentiation with ROS, LOR, FLG, SIRT3, and MnTBAP.
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