Keratin 6a mutations lead to impaired mitochondrial quality control

Running title: Mitochondrial quality control in PC

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Funding sources: IZKF RWTH Aachen University (Start 129/12).
Conflict of interest: The authors state no conflict of interest.

Statements

What’s already known about this topic?

- Terminal epidermal differentiation is a multistep process that includes the elimination of cellular components by autophagy.
- Autophagy-impaired keratinocytes have been shown to result in thickening of epidermal layers.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/bjd.18014
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• Hyperkeratosis also occurs in Pachyonychia congenita, a rare skin disease caused by mutations in keratins 6, 16, and 17.

What does this study add?

• Keratins contribute to mitochondrial quality control as well as maintenance of ER-mitochondria contact sites.
• Keratins influence autolysosomal maturation or reformation.

What is the translational message?

• Overaged mitochondria and autolysosomes accumulate in Pachyonychia congenita.
• Mutations in keratin 6a lead to severely impaired mitophagy which might contribute to Pachyonychia congenita pathogenesis.

Summary

Background: Epidermal differentiation is a multi-level process in which keratinocytes need to lose their organelles including their mitochondria by autophagy. Disturbed autophagy leads to thickening of the epidermis as it is encountered in Pachyonychia congenita, a rare skin disease caused by mutations in keratins 6, 16, and 17.

Objectives: We therefore asked if mitophagy is disturbed in Pachyonychia congenita and if so at which stage.

Methods: Immortalised keratinocytes derived from Pachyonychia congenita patients were used in fluorescence-based and biochemical assays to dissect the different steps of mitophagy.

Results: Pachyonychia congenita keratinocytes accumulate old mitochondria and display a disturbed clearance of mitochondria after mitochondrial uncoupling. Early mitophagy steps and autophagosome formation, however, are not affected. We find that autolysosomes accumulate in Pachyonychia congenita and are not sufficiently recycled.

Conclusion: We propose an influence of keratins on autolysosomal degradation and recycling.

Introduction

Keratin intermediate filaments are present in all epithelial cells. They are critical for stress protection and maintenance of cellular integrity. Equal amounts of type I and type II keratins assemble into cytoplasmic networks that are anchored to desmosomes and hemidesmosomes. Mutations in keratin genes result in more than 30 human diseases, including the rare skin disease Pachyonychia congenita (PC).
PC is caused by mutations in type II keratins 6a, 6b and 6c, and type I keratins 16 and 17. It is characterised by palmoplantar keratoderma and hypertrophic nail dystrophy. Keratin 6, 16 and 17 expression is upregulated when stratified epithelia encounter stressful situations such as wounding and UV exposure.

Recently, intermediate filaments were shown to influence mitochondrial structure, localization and function. Maintaining the cellular mitochondrial pool as healthy as possible is crucial for overall mitochondrial function. Mitochondrial turnover is therefore an essential part of mitochondrial quality control. This is accomplished either by selective elimination of dysfunctional mitochondria through selective macroautophagy (mitophagy) or by renewal of the mitochondrial network through constant fusion and fission of existing mitochondria. Disturbed mitochondrial quality control results in the accumulation of damaged mitochondria that produce less ATP and release high levels of reactive oxygen species (ROS). One of the best-studied mitophagy pathways relies on the serine/threonine kinase phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1) and Parkin. PINK1 is targeted to mitochondria and is usually imported into mitochondria where it is cleaved by presenilin-associated rhomboid-like protein (PARL) and then degraded. Depolarisation of the outer mitochondrial membrane (OMM) results in the stabilisation of PINK1 on the OMM and subsequent recruitment of the E3 ubiquitin ligase Parkin, which attaches ubiquitin moieties to OMM proteins. This, in turn, recruits ubiquitin- and LC3-binding autophagic adaptor proteins such as p62/SQSTM1 (Sequestosome 1) or optineurin in order to induce autophagosome formation around damaged mitochondria. Autophagosomes are transported to and fuse with lysosomes to form autolysosomes. This fusion is required for acidification and the degradation of the autophagosome's cargo. In mammalian cells, the fusion of autophagosomes and lysosomes is controlled by Rab proteins, which belong to the Ras-like GTPase superfamily. One of its members, Rab7, has been implicated in the maturation of autolysosomes. Rab7 has been shown to directly interact with vimentin, modulating vimentin's phosphorylation state and therefore its solubility. Furthermore, disruption of the keratin filament network by treatment of cells with the protein phosphatase inhibitor okadaic acid inhibits autophagy. In order to restore lysosomes, autolysosomes are recycled through a process called autophagic lysosome reformation in which tubules extrude from autolysosomes and form proto-lysosomes that mature into lysosomes.

Epidermal differentiation strongly depends on macroautophagy. While autophagy per se sustains cell health it is also needed for organelle removal during keratinocyte differentiation. This is accomplished by multiple processes including mitophagy. Since PC is characterized by an epidermal hyperproliferation phenotype in conjunction with delayed terminal differentiation, we asked whether PC-linked keratin 6a mutations contribute to a mitophagy defect. Indeed, we could show that mitophagy is impaired due to disturbed autolysosomal reformation.
Materials and methods

Cells

The wild-type human keratinocyte cell line K6a wt was obtained by immortalization of normal human epidermal keratinocytes using the HPV E6/E7 method and was kindly provided by Drs Julia Reichelt, Verena Wally and Thomas Lettner (EB house Austria, Salzburg). The PC cell lines K6aN171K and K6aN171del were derived from Pachyonychia congenita patients also by immortalization of epidermal keratinocytes with the HPV E6/E7 method. They were generously provided by Dr Leonard M. Milstone (Yale University, New Haven).18

All cell lines were cultured in EpiLife Medium (Thermo Fisher) supplemented with 10% (v/v) antibiotic-antimycotic solution (penicillin, streptomycin, Fungizone, Sigma-Aldrich) and 10% (v/v) human keratinocyte growth factor in a 5% CO2 humidified atmosphere at 37°C. They were cultured in uncoated cell culture flasks or on glass coverslips coated with rat tail collagen I (Corning). The cell lines were passaged once or twice per week at a ratio of 1:3. For passaging, cells were washed and incubated for 15 min in PBS without Ca2+/Mg2+ and thereafter incubated for ~10 min with accutase and resuspended in trypsin neutralizer solution (Thermo Fisher). Cells were used within 10 passages. Passages were equal for PC cell lines and 10 higher for wild-type control. For experiments under differentiation conditions, cells were switched from standard culture medium (0.06 mM Ca2+, low calcium) to 1.2 mM Ca2+ medium (high calcium) for three days.

Antibodies

Primary polyclonal rabbit antibody against PINK1 (1:1000 for immunoblot; ab23707) was obtained from Abcam, antibodies against PTEN (1:1000 for immunoblot; 9559) and PI3K (1:1000 for immunoblot; 4292) were from Cell Signaling and antibody against beta-actin (1:2000 for immunoblot; A2066) was from Sigma-Aldrich. Monoclonal rabbit antibody against optineurin (1:1000 for immunoblot; mAb #58981) was obtained from Cell Signaling. Polyclonal guinea pig antibody against p62 (1:1000 for immunoblot; GP62-C) was purchased from Progen Biotechnik. Secondary horse radish peroxidase-coupled antibodies against rabbit (1:5000 for immunoblot; P0448) and against guinea pig (1:5000 for immunoblot; P0141) were obtained from Dako.

Immunoblot analysis

Cell were prepared by first washing them with PBS in the culture dish. 100 µl of 2x SDS buffer (100mM Tris-HCl (pH 8), 10% (v/v) glycerol, 3% (w/v) SDS, 7.5% (v/v) β-mercaptoethanol, 250 µg/ml bromophenol blue) were added and the lysed cells were scraped off with a rubber policeman. Cell lysates were stored at -20°C. Proteins were denatured at 95°C for 5 min and subsequently separated by 8-14% discontinuous sodium dodecyl
sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein transfer onto PVDF membrane was performed in the presence of transfer buffer (25 mM Tris-HCl, 190 mM glycine, 20% methanol) at 100 V for 1 h. The PVDF membrane was blocked with 10% Roti-Block Blocking reagent (Carl Roth) for 1 h. After incubation with the primary antibody diluted in 10% Roti-Block over night at 4°C, the membrane was washed 3x for 10 min with TBS-T (130 mM NaCl, 50 mM Tris base, 0.1% (v/v) Tween-20). The membrane was incubated with the secondary HRP-conjugated antibody diluted in 10% Roti-Block for 1 hour. After repeated washing with TBS-T, visualization of bound antibodies was done with the help of an enhanced chemiluminescence method and detection using the Fusion-Solo.WL.4M. Densitometric evaluation was performed using ImageJ (Fiji distribution, NIH).19,20 To perform incubation of further antibodies on the same membrane, the membrane was incubated in stripping buffer (0.1 M glycine, pH 2) 3x for 20 min to remove the bound antibodies. Subsequent procedures started with a blocking step and continued as described. Three independent experiments were performed.

**Quantitative PCR**

RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. RNA concentration and quality was determined using a NanoDrop1000 (Thermo Fisher). A260/280 ratios were between 1.74 and 2.1. Reverse transcription into cDNA was performed using Transkriptor First Strand cDNA Synthesis kit (Roche) according to the manufacturer’s protocol. Quantitative real time PCR was performed using the FastStart Essential DNA Probes Master kit (Roche) according to the manufacturer’s protocol on a LightCycler 96 Real-Time PCR System (Roche). The cDNA was diluted 1:5 in H2O and a standard was prepared by pooling cDNA of every sample and a dilution series was generated (1:2, 1:4, 1:8, 1:16, 1:32).

The following primers were used: HPRT (UPL probe #022) forward (5’-TGATAGATCCATCTCATGACTGTAGA-3’) and reverse (5’-CAAGACATTTCTTTCCAGTTAAAGTGTG-3’), PINK1 (UPL probe #65) forward (5’-GCCATCAAGATGATGTGGAAC-3’) and reverse (5’-GACCAGCTCTGGCTCATT-3’), PARL (UPL probe #17) forward (5’-GCTCACTGCGGTCTAAACC-3’) and reverse (5’-CTGAATCCCGATTTTGTGG-3’), PARK2 (Parkin) (UPL probe #85) forward (5’-GGAGCTGAGGAATTGACTGGA-3’) and reverse (5’-ACATGTGAATGTCCTTGCT-3’), p62 (UPL probe #14) forward (5’-AGCTGCCTGTGACCCACATC-3’) and reverse (5’-CAGAGAAGGCCCATGGACAG-3’), optineurin (UPL probe #72) forward (5’-AACAGTGACCCCTGACGAAAGG-3’) and reverse (5’-AACAGTGACCCCTGACGAAAGG-3’) and reverse (5’-AAGTTGGGTTTCAAGAAGGCCTA-3’), HDAC6 (UPL probe #58) forward (5’-AGTTCCACTTGACCCAGAC-3’) and reverse (5’-GACCAGACCTACCTGGCTC-3’). RNA from three independent experiments was analysed in duplicates for each condition.

For quantification, efficiency and CT values were determined using LightCycler 96 software (Roche). Fold factor is given as $E_{\text{goi}}(\Delta\text{CT}_{\text{goi}})/E_{\text{ref}}(\Delta\text{CT}_{\text{ref}})$. (GOI = gene of interest; REF = reference gene).
**MitoTimer**

Cells were seeded on day 0 on glass-bottom dishes (12 mm glass-diameter, thickness 1.5#, MatTek) coated with rat tail collagen I (Corning). After two days cells were transfected with the pMitoTimer Addgene plasmid #52659 (gift from Zhen Yan) and were imaged three or four days after seeding. Live cell imaging was done on a Zeiss LSM710 Duo microscope at 37°C. The 488 nm line of an argon/krypton laser was used for fluorescence recording via a 63×/1.40-N.A. DIC M27 oil immersion objective. The emitted light was monitored between 500-540 nm (green signal) and 580-640 nm (red signal) with a pinhole set at 1–2 AU (airy unit) and a laser intensity of 0.2%. Using ImageJ (Fiji distribution, NIH), green and red channels were thresholded (Otsu algorithm) and a mask of regions of interest was calculated that shows signal positive in either channel. Fluorescence intensity mean values were determined for every region of interest. Three independent experiments were performed with 30 cells each per condition. Data were normalized to untreated K6a wild-type cells.

**Induction of mitophagy**

Cells were seeded on collagen-coated coverslips on day 0 and transfected with a Parkin-eGFP construct (provided by Sven Geisler, Tübingen, Germany) on day 1 using Xfect according to the manufacturer’s protocol (Takara Bio). On day 2 the cells were treated with 10 µM CCCP at 37°C for 2 h and 18 h or with DMSO only for 18 h as a control. After 2x 15 min washing with medium at 37°C, cells were stained with 100 nM MitoTracker Red CMXRos (Thermo Fisher Scientific) for 30 min at 37°C. After fixation with 4% warm PFA in diethylpyrocarbonate water for 25 min at RT, nuclei were stained with Hoechst33342 (Invitrogen) and the coverslips were mounted with Mowiol (Carl Roth). Using the ApoTome.2 microscope (Carl Zeiss) cells were imaged at 63x magnification using the 63×/1.40-N.A. DIC M27 oil immersion objective (Carl Zeiss). Imaging of one experiment was performed on the same day for all conditions, with equal exposure times for interference contrast, MitoTracker and Parkin-eGFP. Using ImageJ (Fiji distribution, NIH), the mitochondrial area per cell was determined for Parkin-positive cells after background substraction (rolling ball set to 5). Parkin distribution was scored as cytoplasmic or dotted. Three independent experiments were performed with ≥48 cells per condition.

**LC3**

Cells were seeded at day 0 on collagen-coated coverslips. They were transfected on day 3 with the pftLC3 Addgene plasmid #21074 (gift from Tamotsu Yoshimori).21 On day 4 the low calcium medium was replaced with high calcium medium (1.2 mM Ca$^{2+}$) and cells were further propagated for three days prior to fixation with 4% PFA in DEPC water for 25 min at RT. Nuclei were stained with Hoechst33342 (Invitrogen) and the coverslips were mounted with Mowiol (Carl Roth). Using the ApoTome.2 microscope (Carl Zeiss), ≥16 transfected cells per treatment were imaged with an 63×/1.40-N.A. DIC M27 oil immersion objective

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(Carl Zeiss) with equal exposure times for interference contrast, green and red channels. Using ImageJ (Fiji distribution, NIH), LC3 positive punctae were automatically determined in the green and red channels with the help of the “analyse particles” function (size 5-50 Px) after background subtraction (rolling ball set to 20). Particles positive in only one channel were scored as either green or red and particles positive in both channels were scored as yellow. Three independent experiments were performed with ≥ 16 cells per condition.

Electron microscopy

Pelleted cells were fixed in 4% formaldehyde/1% glutaraldehyde for 2 h and in 1% OsO4 for 1 h. Fixed samples were treated with 0.5% uranyl acetate in 0.05 M sodium maleate buffer, pH 5.2, for 2 h in the dark and thereafter dehydrated and embedded in araldite using acetone as the intermedium. Polymerization was performed at 60°C for 48 h. Semi- and ultrathin sections were prepared with an ultramicrotome (Leica) using a diamond knife. To enhance contrast, sections were treated with 3% uranyl acetate for 5 min and with 0.08 M lead citrate solution for 3 min. Images were taken on an EM10 (Carl Zeiss) with a digital camera (Olympus) using iTEM software (Olympus). Analyses were done on 50000x magnification images. Using ImageJ (Fiji distribution, NIH), ≥50 mitochondria per cell line were encircled to calculate their circumference. Close ER membranes were defined as no more than 15 pixels away from the mitochondrial membrane and their length was determined to calculate the ratio of ER-covered mitochondrial circumference versus whole mitochondrial circumference.

Statistical analysis

Each experiment was performed at least 3 times. The number of analysed cells are indicated for each experiment. Differences between groups were evaluated using GraphPad Prism 5.0 (GraphPad Software) using one-way ANOVA with either Dunnett’s or Bonferroni post-test. Data are summarized and presented as mean ± SD. Differences were considered significant when p ≤ 0.05.

Results

Old mitochondria accumulate in Pachyonychia congenita keratinocytes

To investigate PC pathology, immortalized keratinocytes were prepared from PC patients and healthy individuals by viral E6/E7 transformation. For the current study two PC-derived cell lines producing either K6aN171K or K6aN171del mutants were examined together with a cell line prepared from a healthy donor producing only wild-type K6a (K6a wt). To find out whether mitochondrial quality control is compromised in PC keratinocytes, cells were transfected with MitoTimer. MitoTimer is a mitochondria-targeted dsRed derivative whose fluorescence shifts from green to red over time as the protein ages. Aging of MitoTimer
takes approximately 48h upon its expression. Differences in red/green fluorescence intensity ratios could not be observed 24h after transfection comparing wild-type control and patient-derived cells. However, fluorescence microscopy revealed a significant shift towards red of MitoTimer two days after transfection in both PC patient-derived cells but not in wild-type control cells (Fig. 1). We therefore conclude that mitochondria become older in PC than in control cells.

**Mitochondria-ER contact sites are reduced in Pachyonychia congenita**

Electron microscopy was performed next to examine mitochondrial ultrastructure. No obvious difference in mitochondrial morphology could be identified at first glance (Fig. 2b-d). More careful analysis, however, revealed striking differences in ER-mitochondrial arrangement. While 22.9% of the circumference of mitochondria in control cells were covered by endoplasmic reticulum, only 9.8% were covered in K6aN171K and 8% in K6aN171del PC patient cell lines (Fig. 2a).

Mitochondria-ER contact sites are known as mitochondria-associated membranes (MAMs) and are implicated in mitochondrial quality control. Loss of MAMs leads to reduced mitophagy resulting in accumulation of old and dysfunctional mitochondria. 23

**Expression of early mitophagy related proteins is not altered in Pachyonychia congenita keratinocytes**

Mitophagy comprises a series of events starting with the accumulation of PTEN-induced kinase 1 (PINK1) on dysfunctional mitochondria and subsequent recruitment of the ubiquitin-ligase Parkin. Under normal conditions, PINK1 is imported into mitochondria and cleaved by mitochondrial presenilin-associated rhomboid-like protein (PARL). Upon mitochondrial dysfunction, PINK1 accumulates on mitochondria due to its autophosphorylation on two known phosphorylation sites. 24

Cells were either grown in low calcium medium (0.06 mM Ca$^{2+}$) or in high calcium (1.2 mM Ca$^{2+}$) medium for 3 days to induce differentiation. During differentiation, keratinocytes undergo a series of changes including the loss of their mitochondria by mitophagy.

Quantitative PCR analysis revealed no significant changes in PINK1, PARL and Parkin mRNA expression in either low or high calcium medium (Figs 3a-c). Similarly, PINK1 protein expression was unaltered (Fig. 3d), while PTEN and its counterpart PI3K were reduced in PC cells independently of calcium addition (Figs 3e, f), although their ratios were unaltered. These results suggest that recognition and marking of dysfunctional mitochondria for subsequent mitophagy are not disturbed.
Mitophagy is impaired in Pachyonychia congenita keratinocytes

Although PINK1 is present in both PC cell lines, old mitochondria still accumulate and are not disposed of. We therefore asked if Parkin is recruited to mitochondria following PINK1 stabilisation on the outer mitochondrial membrane. To this end, healthy control and PC cells were transfected with fluorescent Parkin to visualize its translocation. Mitophagy was induced by adding the mitochondrial uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP). CCCP treatment led to the translocation of Parkin to mitochondria within 2 hours (Fig. 4a). Mitochondria were eliminated by mitophagy after 18 hours (Fig. 4a) in control cells. Untreated cells showed an even cytoplasmic distribution of Parkin-eGFP (100% of control cells, 99.35% of K6aN171K PC cells, and 100% K6aN171del PC cells) (Figs 4a-c upper panels; quantification in Fig. 4d). 2 hours after mitophagy induction by CCCP, 93.77% of control cells and 95.33% of K6aN171K PC cells and 94.60% of K6aN171del PC cells showed a dotted Parkin-eGFP pattern co-localising with MitoTracker staining (Figs 4a-c middle panels; quantification in Fig. 4d). Hence, Parkin recruitment to dysfunctional mitochondria is not affected by K6a mutations. 18 hours after mitophagy induction by CCCP, 72.61% of the control cells showed a restored steady state cytoplasmic Parkin pattern and lost their mitochondria. In contrast, only 9.33% of K6aN171K PC cells and 9.8% of K6aN171del PC cells displayed a cytoplasmic Parkin pattern again and lost their mitochondria while the remaining fraction of cells still had Parkin recruited to their mitochondria (Figs 4a-c lower panels; quantification in Fig. 4d). Accordingly, the percentage of the area of mitochondrial signal per cell was not reduced in K6aN171del while K6aN171K and wild-type controls showed less mitochondrial signal (Fig. 4e). Of note, in K6aN171K cells the mitochondrial signal decline only occurred within the first 2 hours of CCCP treatment whereas in K6a wt a reduction could be observed between all time points. This leads to the conclusion, that while dysfunctional mitochondria are marked for mitophagy and Parkin is recruited to mitochondria, subsequent steps of mitophagy are disturbed in PC.

Markers for autophagosome formation are not altered in Pachyonychia congenita keratinocytes

To further narrow down which step of mitophagy is impaired in PC we investigated the expression of mitophagy and autophagosome markers. After translocation of Parkin, OMM proteins become highly ubiquitinylated which is recognized by the ubiquitin-binding histone deacetylase 6 (HDAC6) and the autophagy receptors SQSTM1/p62 and optineurin. p62 mediates the aggregation of dysfunctional mitochondria and directly binds to the autophagic effector proteins LC3A and LC3B. Optineurin becomes recruited to damaged mitochondria marking the site of initial autophagosome formation. HDAC6 promotes autophagy by mediating the attachment of autophagosomes to dynein motors for transport to lysosomes. Furthermore, HDAC6 induces cortactin-dependent actin-remodelling to facilitate autophagosome-lysosome fusion.
Optineurin, HDAC6 and p62 mRNA expression was not changed in healthy control cells as well as in PC keratinocytes after induction of differentiation by calcium addition (Figs 5a-c). Accordingly, optineurin and p62 were unaffected at the protein level (Figs 5d-e). Therefore, differential expression of key players of autophagosome formation and maturation are most likely not the cause for disturbed mitophagy in PC keratinocytes.

Autolysosome maturation is perturbed in Pachyonychia congenita keratinocytes.

We next asked whether autolysosome formation is affected in PC keratinocytes. The process of autolysosome formation can be monitored by using a LC3 tandem fluorophore. LC3 specifically localises to autophagic membranes of autophagosomes and autolysosomes. The fusion protein fluoresces both green and red under steady-state conditions. After induction of mitophagy mRFP-GFP-LC3-containing autophagosomes fuse with lysosomes to form autolysosomes and lose their green fluorescence due to lysosomal acidic and degradative conditions. The total number of autolysosomes per cell was already elevated under steady-state low calcium conditions in K6aN171K and K6aN171del keratinocytes compared to healthy control keratinocytes (61.99 autolysosomes/cell in control cells, 105.6 autolysosomes/cell in K6aN171K PC cells, and 80.88 autolysosomes/cell in K6aN171del PC cells) (Figs 6a-c upper panels; quantification in Fig. 6e). While calcium addition did not alter the amount of autolysosomes in control cells, the number of autolysosomes per cell was significantly increased in both PC cell lines (69.65 autolysosomes/cell in control cells, 138.00 autolysosomes/cell in K6aN171K PC cells, and 152.5 autolysosomes/cell in K6aN171del PC cells) (Figs 6a-c lower panels; quantification in Fig. 6e). Likewise, while the percentage of autolysosomes (only red LC3 puncta) was not altered in low calcium conditions compared to wild-type control cells, the elevation of extracellular calcium led to a significantly increased percentage of autolysosomes in PC cells (57.86% vs. 56.82% in control cells, 55.09% vs. 71.13% in K6aN171K PC cells, and 63.8% vs. 82.6% in K6aN171del PC cells) (Fig. 6d).

Taken together, these results indicate that autolysosome generation is unaffected but that autolysosomal recycling is severely impaired in cells derived from Pachyonychia congenita patients.

Discussion

In this paper we addressed the possible role of mitophagy in the pathogenesis of Pachyonychia congenita. To this end we used two patient-derived keratinocyte cell lines with different mutations in keratin 6a: K6aN171K and K6aN171del. We show that in PC old mitochondria accumulate over a time period of 48 hours and that CCCP-induced mitochondrial clearance is delayed. Still, early and late mitophagy markers are not changed. While autolysosome formation is not affected, autolysosomes accumulate because of compromised maturation or recycling, suggesting a role of keratin 6a in autolysosomal turnover.

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Autophagy is a conserved process which leads to the lysosomal degradation of cellular components such as proteins and organelles. Elimination of old or dysfunctional mitochondria via autophagy is called mitophagy. Disturbed mitophagy and autophagy lead to a number of human diseases such as cardiomyopathies, and neurodegenerative and skin diseases. Functional autophagy is especially important in terminal differentiation of keratinocytes who lose their organelles during the course of keratinization to become dead corneocytes in the outermost epidermal layer. Accumulation of autolysosomes could only be seen after calcium-induced differentiation of PC keratinocytes. There have been several reports which confirm the role of autophagy in keratinocyte differentiation. For example, mice lacking ATG7, which is important for autophagosome assembly, have a low level of autophagy and show epidermal acanthosis and hyperkeratosis. Keratinocytes treated with the autophagy-inhibitors 3-MA or wortmannin present enhanced inflammatory responses and increased cell proliferation. Furthermore, autophagy-deficient keratinocytes display a weakened stress response resulting in DNA damage, cell cycle arrest and premature aging.

Autophagy is vital for cells to survive stresses as it eliminates damaged proteins and organelles and thus restores cell health. Part of the stress response in keratinocytes after wounding is the upregulation of keratins 6, 16 and 17. Keratin 6 has been linked to cell migration and wound healing. While not much is known about the influence of autophagy on wound healing so far, it was shown that ATG7 knockdown stimulated cell migration while inhibition of MTORC1 by rapamycin enhanced autophagy and reduced cell migration.

A role of intermediate filaments in mitochondrial homeostasis has been pointed out in several reports (reviewed in), but no clear functional connection has been established yet. For example, it has been shown that desmin mutations lead to a disturbed mitochondria-ER distance. Mitochondria-ER contact sites are important for the formation of the omegasome, a membranous structure that engulfs damaged mitochondria in order to form autophagosomes. Although mitochondria-ER contact sites were reduced in PC patient cells, autophagosome formation seemed to be unaffected. Additionally, MAMs are specialized domains for intracellular calcium transfer, shuttling calcium from ER to the mitochondrial matrix. Intracellular calcium acts as a regulator of autophagy by inhibiting MTORC1 and therefore activates autophagy. Loss of mitochondria-ER contact sites as well as a decrease in mitochondrial calcium and an increase in cytoplasmic calcium leads to autophagy. Elevation of intracellular calcium of keratinocytes additionally induces their differentiation, explaining why the effects of PC mutations on autophagy can be seen after calcium treatment. Keratin 6a mutations may act on two different levels (i) by disturbing mitochondria-ER calcium handling, which results in enhanced autophagy, and (ii) by disturbing autolysosomal recycling.

Autolysosomal reformation is the process in which lysosomal components of autolysosomes are recycled in order to reform lysosomes. During this reformation tubules protrude from the autolysosomal membrane and form protolysosomes which initially lack lysosomal acidity. Those tubules then become acidic and mature into lysosomes. Rab7, a vesicle transport regulator, and KIF5B, a member of the kinesin 1 family, are two important factors for autolysosomal recycling. KIF5B has been implicated in the control of intermediate
filament localization in heart muscle while Rab7 has been shown to directly interact with the intermediate filament vimentin.\textsuperscript{13,42} Additionally, vimentin as well as keratin intermediate filaments have been shown to move along microtubules with the help of KIF5B.\textsuperscript{43} Furthermore, it has recently been shown that autophagy and lysosomal functions play an important role in keratinocyte differentiation in organotypic human skin where lysosomes promote mitochondrial metabolism and the associated production of mitochondrial ROS, which in turn triggers autophagy upon its release into the cytoplasm of suprabasal keratinocytes.\textsuperscript{44}

Autolysosomal reformation is upregulated during stress situations. It depends on mTOR signalling and can be blocked by the mTOR inhibitor rapamycin.\textsuperscript{15,45} In contrast, rapamycin treatment also induces autophagy providing a feedback mechanism to avoid excessive cell-harming autophagy.\textsuperscript{45} Rapamycin furthermore blocks Krt6a expression in human keratinocytes and ameliorates PC symptoms.\textsuperscript{46}

Here, we show for the first time that mitophagy and autolysosomal turnover are disturbed in PC patient’s keratinocytes. Further investigations clarifying the interplay between autophagy and epidermal differentiation are necessary to understand PC pathogenesis.

**Acknowledgments**

We are especially grateful to the Pachyonychia congenita community and Leonard M. Milstone, for kindly providing the used cell lines. We thank the EB house Austria for the wild-type cell line. We thank Sven Geisler, Tamotsu Yoshimori and Zhen Yan for plasmids. Research was funded by IZKF RWTH Aachen University (Start 129/12).
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**Figure legends**

**Figure 1. Old mitochondria accumulate in Pachyonychia congenita keratinocytes.**

Cells were transfected with MitoTimer encoding a mitochondria-targeted dsRed derivative whose fluorescence shifts from green to red with increasing time after synthesis. Fluorescence images were recorded 24 and 48 hours after transfection. (a-c) show representative fluorescence micrographs taken from wild type K6a and mutant K6aN171K and K6aN171del keratinocytes 48h after transfection. Each micrograph depicts an overlay of the green and red channel. The histogram shows the ratiometric quantification of three independent experiments of 30 cells per cell line each (d). Ratios were normalized to control K6a wt cells 24 hours after transfection. MitoTimer changes its colour from green to red over the course of 48 hours. No differences in red/green ratios could be observed 24 hours after transfection. In contrast, after 48 hours K6aN171K and K6aN171del keratinocytes display a significantly increased red/green ratio of fluorescence intensity compared to K6a wt cells (d). Scale bars = 10 µm. Comparisons to 24 hours after transfection K6a wt cells were performed using one-way ANOVA with Dunnett’s post-test. * p<0.05, *** p<0.001.

**Figure 2. Mitochondria-ER contact is reduced in Pachyonychia congenita keratinocytes.**

Electron microscopy was performed to investigate the spatial relationship between mitochondria and ER. (a) shows a histogram of the quantitative analysis of ER contact with mitochondria in K6aN171K, K6aN171del and control cells (n ≥ 50 mitochondria per cell line). (b-d) present selected electron micrographs depicting examples of mitochondria-ER contact sites in K6a wt (b), K6aN171K (c) and K6aN171del (d) keratinocytes (indicated by arrowheads). Note the significant reduction of contact sites in the mutant cells (b-d). Scale bars = 100 nm. Comparisons between groups were performed using one-way ANOVA with Bonferroni post-test. *** p<0.001.

**Figure 3. Expression levels of early mitophagy markers are not affected by K6a mutations.**

(a-c) The histogram depicts the results of qPCR (three independent experiments each; mean ±SD) determining the mRNA levels of the early mitophagy markers PINK1 (a), PINK1-
cleaving protease PARL (b) and Parkin (c) in K6a wt and mutant K6aN171K and K6aN171del keratinocytes which were grown under low and high calcium conditions. No significant alterations are detectable. (d-f) Results of immunoblot analysis of lysates prepared from the same cell lines (top: examples of immunoblot images; bottom: histograms depicting the results of three independent experiments; mean ±SD) reveal that PINK1 protein levels are also not significantly altered (d) but that its inductor PTEN is slightly decreased (e) and that the PTEN antagonist PI3K is significantly decreased in K6aN171K cells under low and high calcium conditions and in K6aN171del keratinocytes under high calcium conditions compared to K6a wt. Comparisons between groups were performed using one-way ANOVA with Bonferroni post-test. * p<0.05.

Figure 4. Mitophagy is severely impaired in Pachyonychia congenita keratinocytes.

Keratinocytes were transfected with a fluorescent Parkin reporter (Parkin-eGFP) one day after seeding and treated with the mitophagy inducer CCCP (10 µM) for 2 h and 18 h or with DMSO as a control for 18 h, stained with MitoTracker and subsequently fixed. (a-c) show representative fluorescence micrographs of three independent experiments with at least 48 cells per condition each. (d) shows a histogram of the percentage (mean ± SD) of cells displaying either dotted or diffuse cytoplasmic Parkin. All cell lines display an even cytoplasmic Parkin distribution under control conditions (a-c; d), shifting to dotted, i.e. mitochondrial-targeted Parkin after two hours of CCCP treatment as expected for dysfunctional mitochondria. After 18 h of CCCP treatment, however, only 72.61% of K6a wt keratinocytes display a cytoplasmic Parkin distribution again (a, e), whereas 90.67% of K6aN171K and 90.2% of K6aN171del cells still display dotted Parkin (b, c, e). (e) The histogram depicts the percentage (mean ± SD) of the cell area covered by mitochondria. Note that mitochondrial area is significantly reduced in K6a wt cells by CCCP (4.87% in untreated cells, 2.84% after 2 h of CCCP, 0.58% after 18 h of CCCP) but that mitochondrial area in PC cell lines only changes within 2 h of CCCP treatment but not further after 18 h CCCP treatment. Scale bars = 20 µm. Comparisons between groups were performed using one-way ANOVA with Bonferroni post-test. * p<0.05, ** p<0.01, *** p<0.001.

Figure 5. Expression levels of markers for autophagosome formation are not affected by K6a mutations.

(a-c) Histograms of autophagosomal mRNA levels (mean ± SD) were prepared from qPCR analyses of three independent experiments from cells grown at low and high calcium conditions. Neither p62 (a) nor optineurin (b) or HDAC6 (c) show significant alterations of mRNA levels in mutant K6aN171K and K6aN171del compared to K6a wt keratinocytes. (d-e) Immunoblot analyses of cell lysates from the same cell lines further demonstrate that p62 (d) and optineurin (e) protein levels are also not altered in the mutant cell lines. Corresponding histograms of the protein levels (mean ±SD; three independent experiments)
are shown below. Comparisons between groups were performed using one-way ANOVA with Bonferroni post-test.

Figure 6. K6a mutations lead to impaired autolysosome maturation and recycling.

To monitor autophagosome and autolysosome formation cells were transfected with a LC3 tandem fluorophore. Autophagosomes are represented by green and red puncta, whereas autolysosomes show only red fluorescence. (a-c) The representative fluorescence micrographs (three independent experiments of n≥16 cells per condition each) taken from keratinocytes grown at low and high calcium conditions show that the total number of only red puncta (autolysosomes) is significantly increased in PC cell lines compared to K6a wt cells grown at high calcium (histogram in d). Furthermore, the percentage of only red puncta is significantly increased at high calcium compared to low calcium conditions in both mutant cell lines but not in wt K6a keratinocytes (quantification in e). Scale bars = 20 µm. Comparisons between groups were performed using one-way ANOVA with Bonferroni post-test. * p<0.05, ** p<0.01, *** p<0.001.