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RESEARCH NOTE

Expression of the retina-specific flippase, ABCA4, in epidermal keratinocytes [version 1; peer review: 2 approved with reservations]

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Abstract

ATP-binding cassette, sub-family A, member 4 (ABCA4) is a photoreceptor transmembrane protein that is responsible for flipping N-retinylidene-phosphatidylethanolamine, a key intermediate in the visual cycle, from the lumen to the cytoplasmic leaflet of photoreceptor outer segment disks. Mutations in *ABCA4* cause a build-up of toxic retinoids resulting in a variety of retinal degenerative phenotypes, including Stargardt disease, cone-rod dystrophy and retinitis pigmentosa. Since many of the *ABCA4* variants are rare and non-exomic, their pathogenicity is often difficult to demonstrate statistically. Given that the neural retina is inaccessible to molecular analysis in living patients, we use patient-specific induced pluripotent stem cell (iPSC)-derived retinal neurons to identify and model disease-causing mutations. Here we demonstrate that a truncated version of the retinal-specific transmembrane enzyme ABCA4 is expressed in epidermal keratinocytes and is required for cellular proliferation and viability at late passage. This finding is of great importance for labs that wish to investigate the pathophysiology of novel *ABCA4*-variants, without having to incur the added expense and scientific expertise associated with iPSC generation, culture and differentiation. Likewise, this finding is also important for those intending to generate iPSCs from patient specific keratinocytes, which can prove difficult when *ABCA4* mutations are present.

Keywords

ABCA4 , induced pluripotent stem cells , keratinocytes

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Any reports and responses or comments on the article can be found at the end of the article.

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Competing interests: No competing interests were disclosed.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Narrative

We have demonstrated that a truncated isoform of the retina-specific protein, ABCA4 is expressed in human epidermal keratinocytes. This isoform is required for cell viability at late passage and as such can be used to interrogate the pathophysiology of novel *ABCA4* mutations.

Introduction

ATP-binding cassette, sub-family A, member 4 (ABCA4, previously named ABCR for its retina-specific expression) is a transmembrane protein that is primarily localized to retinal photoreceptors¹ where it is responsible for flipping N-retinylidene-phosphatidylethanolamine, a key intermediate in the visual cycle, from the lumen to the cytoplasmic leaflet of photoreceptor outer segment disks^{1,2}. Mutations in *ABCA4* cause a build-up of toxic retinoids resulting in a variety of retinal degenerative phenotypes, including Stargardt disease, cone-rod dystrophy and retinitis pigmentosa². To date over 400 different mutations in *ABCA4* have been reported³. Since many of these variants are rare and non-exomic, their pathogenicity is often difficult to prove⁴. As the primary focus of our group is to develop gene and autologous cell replacement-based treatments for rare inherited retinal degenerative diseases, unambiguous identification of disease-causing mutations is essential.

The neural retina is inaccessible to molecular analysis in living patients. As such, to determine if newly identified mutations in retina-specific genes are disease causing, we use patient-specific induced pluripotent stem cell (iPSCs)-derived retinal neurons⁵. Typically, iPSCs are generated in our lab using patient-specific keratinocytes isolated from the epidermis of 3 mm punch biopsies. Briefly, the epidermis is separated from the underlying dermis via dispase incubation, and keratinocytes are liberated via trituration. Keratinocytes are subsequently maintained on collagen-coated culture plates in Epilife medium (Gibco; Life Technologies) supplemented with 1% human Keratinocyte Growth Supplement (Gibco). While generating iPSCs from patients suspected of having *ABCA4*-associated retinal disease, we noticed that primary keratinocyte proliferation was often slow and reprogramming efficiencies were low compared to age-matched controls, suggesting that *ABCA4* mutations affect keratinocyte physiology directly. In this study a series of western blot, immunocytochemistry, and cytometry experiments were performed to demonstrate that ABCA4 is expressed in human keratinocytes and that *ABCA4* mutations alter cellular viability.

Results

Dataset 1. Raw data for 'Expression of the retina-specific flippase, ABCA4, in epidermal keratinocytes'

<http://dx.doi.org/10.5256/f1000research.8089.d114077>

README.txt contains a description of the files.

To determine if the ABCA4 protein was expressed in keratinocytes, a Western blot comparing lysates isolated from normal human, bovine and murine retina, murine retina with *Abca4* deletions, and human keratinocytes was performed. Keratinocytes from three different control individuals of three different ages expressed a truncated protein that corresponded in size to an alternatively

spliced 70 kDa ABCA4 isoform (detected previously via rt-PCR⁴; (Figure 1A)). Immunocytochemical labeling using an antibody targeted against ABCA4 demonstrated a perinuclear pattern of labeling (Figure 1B–C). To determine if mutations in *ABCA4* affect cellular proliferation and/or senescence, keratinocytes from three control, three retinal disease patients without *ABCA4* mutations, and three patients with molecularly confirmed *ABCA4*-associated retinal disease were analyzed using propidium iodide and a Tali image based cytometer (Figure 1D). For this analysis, keratinocytes received fresh medium every other day and were passaged routinely once a week. Cell viability readings were assessed at each cell passage. A significant decrease in cell viability was detected between passages 6–9 in cells isolated from patients with *ABCA4*-associated disease as compared to normal and non-*ABCA4* disease controls (Figure 1D). Finally, immunocytochemical staining of normal human skin shows robust ABCA4 expression throughout the keratinizing layer (Figure 1E).

Conclusions

Taken together, these data demonstrate that a truncated version of the retina-specific transmembrane enzyme ABCA4 is expressed in epidermal keratinocytes and is required for cell viability at late passage. Although future studies will be needed to elucidate the exact function of ABCA4 in the skin, this finding may be useful for individuals attempting to determine the pathogenicity of novel mutations in the *ABCA4* gene.

Materials and methods

Ethics

All patients provided written, informed consent for this study, including skin biopsies and use of keratinocytes to generate induced pluripotent stem cells, which was approved by the Institutional Review Board of the University of Iowa (project approval #199904167) and adhered to the tenets set forth in the Declaration of Helsinki.

Western blotting

Western blotting was performed as previously described^{5–7}. Briefly, 40 µg of protein lysate from unaffected human donor retina (Iowa Lions Eye Bank, Coralville, IA), bovine retina (Bud's Custom Meats Inc., Riverside, IA), control mouse retina (C57Bl/6J; The Jackson Laboratory, Bar Harbor, ME; Cat. No. 00064), *Abca4*^{-/-} (KO; a kind gift from Dr. Gabriel Travis, University of California Los Angeles), Japanese Fancy mouse retina (a kind gift from Dr. Toshihiko Shiroishi, National Institute of Genetics, Mishima, Japan) and keratinocytes from three individuals ages 32, 54 and 68 were separated via SDS-PAGE on a 4–20% gradient gel, blotted and labeled with a mouse monoclonal anti-ABCA4 antibody at a 1:500 dilution (Santa Cruz Biotechnology, Dallas, TX; Cat. No. sc-65672). Bands were visualized using SuperSignal® West Pico Chemiluminescence Substrate (Thermo Fisher Scientific, Waltham, MA; Cat. No. 34080) and autoradiography. Images of the final blots were captured with an iPhone 6S (Apple, Cupertino, CA).

Immunocytochemistry

Keratinocytes were fixed for 10 minutes in 4% paraformaldehyde in 1X phosphate buffered saline (PBS), rinsed in 1X PBS and blocked in immunocytochemical blocking buffer [1X phosphate buffered

Figure 1. ABCA4 is expressed in keratinocytes and human skin and is required for cell viability. **A**) Western blot of human (Hmn Ret), bovine (Bov Ret) and murine (Ms Ret) whole retinal lysate, retinal lysate from two mouse strains lacking *Abca4* (*Abca4* KO and Japanese Fancy (JF1)) and keratinocytes from three individuals 32, 54 and 68 years of age (KTC 32, 54, and 68, respectively). Robust ABCA4 expression is observed at 250 kDa in human, cow and mouse retina, but is completely absent in each *Abca4* knockout retina. Keratinocytes from each control individual express a 70 kDa isoform of ABCA4. **B–C**) Immunocytochemical labeling of control KTC 32 (**B**) and control KTC 54 (**C**) patient keratinocytes with an anti-ABCA4 antibody (red) and a filamentous actin stain (Phalloidin; green). A perinuclear pattern of ABCA4 labeling was detected. **D**) Keratinocyte viability was assessed at passages 6–9 by staining cells with propidium iodide. Keratinocytes assessed from three different individuals with molecularly confirmed mutations in *ABCA4* (“*ABCA4*”) are much less viable compared to control keratinocytes (“control”) and cells from three different patients with non-*ABCA4*-associated retinal disease (“Non-*ABCA4*”). **E**) Immunocytochemical staining of control human skin with the same anti-ABCA4 antibody used in Figure 1A–C above showing ABCA4 labeling (green) throughout the keratinizing layer of the epidermis. Scale bars = 100 µm (**B–C**) and 40 µm (**E**).

saline (Cat. No. 10010-023; Thermo Fisher Scientific, Waltham, MA, USA), 3% bovine serum albumin (Cat. No. A30075-100.0; Research Products International Corp., Mount Prospect, IL, USA), 5% normal goat serum (*Note: another species can be substituted here to better suit the secondary antibody species*; Cat. No. 5425; Cell Signaling, Danvers, MA, USA), 0.5% Triton X-100 (Cat. No. T8787; Sigma-Aldrich, St. Louis, MO, USA), and 0.2% NaN_3 (sodium azide; Cat. No. 438456; Sigma-Aldrich, St. Louis, MO, USA)]. Cells were labeled with mouse monoclonal anti-ABCA4 antibody at a dilution of 1:500 (Santa Cruz Biotechnology, Dallas, TX; Cat. No. sc-65672) and stained with Alexa Fluor® 488 Phalloidin at a dilution of 1:1000 (Life Technologies/Thermo Fisher Scientific, Waltham, MA; Cat#: A12379) to label filamentous actin overnight at 4° Celsius. The following morning, cells were washed using wash buffer [1X phosphate buffered saline (Cat. No. 10010-023; Thermo Fisher Scientific, Waltham, MA, USA), 0.2% Tween® 20 (Cat. No. P2287; Sigma-Aldrich, St. Louis, MO, USA)]. ABCA4 was detected using the goat anti-mouse 555 fluorescently conjugated Alexa Fluor® secondary polyclonal antibody at a dilution of 1:1000 (Life Technologies/Thermo Fisher Scientific, Waltham, MA; Cat#: A-21422) for two hours at room temperature. Cell nuclei were coverslipped and counterstained using Poly(vinyl alcohol) (PVA)-based mounting medium containing 1,4-Diazabicyclo[2.2.2]octane (DABCO) [100 µg/ml PVA (Cat. No. 341584; Sigma-Aldrich, St. Louis, MO, USA), 25% v/v glycerol (Cat. No. G9012; Sigma-Aldrich, St. Louis, MO, USA), 0.1M Tris-HCl, pH 8-8.5, 25 µg/ml DABCO (Cat. No. 290734; Sigma-Aldrich, St. Louis, MO, USA) and 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Cat. No. D9542; Sigma-Aldrich, St. Louis MO, USA; 1:10,000 dilution). Cells were imaged using a Leica DM 2500 SPE confocal microscope (Leica Microsystems, Wetzlar, Germany). Human skin was labeled with anti-ABCA4, secondary antibody and visualized in the same manner.

Cell viability

To determine if mutations in *ABCA4* affect cellular proliferation and viability, keratinocytes from three control, three retinal disease patients without *ABCA4* mutations, and three patients with molecularly confirmed *ABCA4*-associated retinal disease were analyzed using propidium iodide (Thermo Fisher Scientific, Waltham, MA; Cat. No. P3566) and a Tali image-based cytometer (Life

Technologies, Waltham, MA). Keratinocytes were maintained on collagen-coated culture plates in Epilife medium (Gibco/Life Technologies, Waltham, MA; Cat. No. M-EPI-500-CA) supplemented with 1% Human Keratinocyte Growth Supplement (Gibco/Life Technologies, Waltham, MA; Cat. No. S-001-5). Keratinocytes received fresh medium every other day and were passaged routinely once a week. Cell viability readings were obtained using the Tali image-based cytometer via PI staining and 20 image analysis, i.e. at the time of passage a sample from each cell line was loaded into the Tali, 20 separate images were taken and the number of live vs. dead cells and in turn % viability was automatically generated. This data was collected from cell lines obtained from 7 control individuals, 5 patients with non-*ABCA4*-associated retinal disease (disease controls) and 13 patients with two molecularly confirmed disease causing *ABCA4* mutations. To determine statistical significance, a one-way ANOVA with a Tukey's post-hoc test was performed using Graphpad Prism software version 6.0b (Graphpad Software Inc., La Jolla, Ca). A p-value < 0.05 was considered significant.

Data availability

F1000Research: Dataset 1. Raw data for 'Expression of the retina-specific flippase, ABCA4, in epidermal keratinocytes', [10.5256/f1000research.8089.d114077](https://doi.org/10.5256/f1000research.8089.d114077)⁸

Author contributions

LAW, RFM, EMS and BAT conceived the study. LAW, EEK and JAP performed the experiments. LAW, RFM, EMS and BAT wrote and approved the final manuscript.

Competing interests

No competing interests were disclosed.

Grant information

NIH Directors New Innovator Award 1-DP2-OD007483-01 (BAT); NEI EY016822 (EMS); NEI EY024605 (RFM); Research to Prevent Blindness; Stephen A. Wynn Foundation.

I confirm that the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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The manuscript by Wiley *et al.* presents an interesting and novel finding that keratinocytes express a truncated form of ABCA4 protein that may be responsible for cell viability. Keratinocytes from patients with disease-causing mutations in the ABCA4 protein appear to have low viability upon passaging in culture when compared to control cells (from patients with unknown ABCA4 mutations and unaffected individuals). The manuscript is well written and the data are clearly presented. However, there are some important key points that are not fully clear thereby making it difficult to accurately assess the biological relevance of the current work:

- Fig. 1 panel A: an internal control should be considered in order to estimate the relative abundance of the ABCA4 isoform relative to the retina samples. Although the authors do not comment on this aspect, there is a ~70kDA band in both bovine and human retina samples. Perhaps, lowering the total protein amount and including an internal standard (i.e. tubulin, actin or GAPDH) along with keratinocytes from patients with disease-causing ABCA4 mutations could help with interpreting and quantifying the data from this panel.
- Fig. 1 panel B and C: at gross evaluation, there are slight differences between the distribution of ABCA4/Phalloidin staining of the two analyzed normal keratinocytes. There are larger cells and more punctate ABCA4 distribution in KTC 54 (panel B) compared to KTC32 (panel C), possibly due to different passages or culturing conditions. It may help to add the ABCA4/Phalloidin staining profiles of keratinocytes collected from patients with disease-causing ABCA4 mutations. All cells should be harvested, cultured and passaged similarly. Moreover, how specific is this ABCA4 antibody? A negative control, pre-immune (secondary antibody only) must be included to determine the background staining level.
- Fig. 1 panel D: representative images of passages 6 and 9 should be provided for each

group. The identification of disease-causing ABCA4 mutations found in the studied keratinocytes should be reported.

- Fig. 1 panel E: ABCA4 immunostaining should be done in the skin of the patients with molecularly confirmed ABCA4 mutations also not just in the control skin of a normal individual.
- General comment: It would be important to know if selected keratinocytes from patients with disease-causing ABCA4 mutations express a misfolded ABCA4 protein or if they do not express the ABCA4 protein at all. Providing sequencing data regarding the ABCA4 variants from keratinocytes isolated from Stargardt patients would be valuable.

The discussion should be expanded to explain how the identification of this truncated form of ABCA4 is relevant to studies of the full-length ABCA4 in the iPS-derived photoreceptor cells or perhaps in other cell types. It would also be interesting to know if it is even possible to reprogram and differentiate these genetically mutated keratinocytes in order to obtain a specific cell-type that may be useful for future analyses.

The identification of the ABCA4 protein in keratinocytes, most likely an alternatively spliced isoform, is novel and suggests a physiologic role of ABCA4 in other cell types besides the photoreceptors.

Competing Interests: No competing interests were disclosed.

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Author Response 20 Apr 2016

Luke Wiley, University of Iowa, Iowa City, USA

We thank both reviewers for their insightful comments and critique of this brief manuscript. We agree with the comments from each reviewer, particularly the additional experiments that each suggested, which would act to strengthen the paper. However, we felt this was an important observation to share in an open publishing forum, so as to reach as many people as possible. Again, while we agree with and appreciate each reviewer's critique, to address all of these comments would be too time consuming for a brief observational report such as this.

Competing Interests: No competing interests were disclosed.

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The authors present some novel data showing the expression of a truncated version of the ABCA4 protein in primary human keratinocytes. They determine that primary keratinocytes from patients with retinal disease-causing ABCA4 mutations have lower viability at later passage. The article is generally well written and the data is novel and interesting, however I would recommend addressing some points I raise below.

Abstract.

The final sentence a little vague. Without having read the paper this statement is not too clear. As the abstract should draw the reader into the article, a better summarising/concluding statement should end the abstract. Also, the authors state that ABCA4 is important for cellular proliferation. Their paper does not however provide any measure of proliferation so they should only state that viability is affected, in the absence of such data.

Figure 1A

Western blot bands prominent but rather spread-out, particularly in the ms retina. What do the other bands present correspond to, are they non-specific? Other known splice variants? Do the authors know the antibody epitope and are they confident it cross-reacts appropriately with ABCA4 in different species?

Figure 1D

Cells from how many patients were used for each measurement? The figure legend suggests 3, but the materials and methods states different numbers of patients/donors for each condition. This needs clarifying in the figure legend. Also, why no indication of statistical significance on the graphs? The authors claim to have performed one-way ANOVA but don't show the result in the figure?

Figure 1B/C

What was the passage of the cells used to generate these images and are they matched? The image in Figure 1C appears to have many more large, senescent cells in which the staining pattern appears less perinuclear and more punctate, throughout the cytoplasm. Can the authors provide more details and comment on this?

Figure 1E

The DAPI staining appears absent in the basal layer in places, why is this? The description of the staining is somewhat inadequate. What about subcellular localisation? In the basal layer it almost looks membraneous in places, however as you move through the stratum spinosum and granulosum, the staining becomes strongly and uniformly cytoplasmic. This Figure would benefit from additional annotation, showing the dermal-epidermal junction and highlighting the different layers of the epidermis. This would help readers identify the changes in localisation that appear to occur as the keratinocytes differentiate.

Conclusions

Given the authors finding that ABCA4 is expressed in keratinocytes, plus my own previously published findings that it is also strongly expressed in human hair follicles, is it appropriate to still call ABCA4 “retina-specific”? I would say not.

Can the authors expand on exactly how and why this study will be useful for determining the pathogenicity of novel ABCA4 mutations?

Overall – I understand that iPSCs derived from epidermal keratinocytes are used as a retinal surrogate in lieu of being able to access retina’s from living patients. I now understand that keratinocytes express a truncated version of ABCA4, which has some unknown role in maintaining the viability of the isolated primary cells. What is not clear to me is how this can help with studying the pathogenicity of ABCA4 mutations? If only a truncated version of ABCA4 is present in keratinocytes, how does this form relate to the full-length protein? And can the mutations that are commonly found in patients with retinal disease be mapped to this truncated version? Overall I think this is really interesting phenomenological data, but for me the authors do not clearly describe how their findings could be useful for studying novel mutations in full-length ABCA4, as would be present in the retina? It would be really useful to have some sequencing data that might show differences in the truncated ABCA4 present in the primary keratinocytes from control vs. stargardt patients.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 20 Apr 2016

Luke Wiley, University of Iowa, Iowa City, USA

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Competing Interests: No competing interests were disclosed.

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