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Non-Visual Opsin 3 Localizes with Microtubules During Mitosis and is Essential for Cytokinesis

F. Labarrade[1], C. Gondran*[2], K. Cucumel[3]

[123]Ashland Specialties France, Global Skin Research Center 655 route du Pin Montard, 06904 Sophia Antipolis, France.
flabarrade@ashland.com, cgondran@ashland.com, kcucumel@ashland.com

***Corresponding Author:** Dr. Catherine Gondran, Ashland Specialties France, Global Skin Research Center 655 route du Pin Montard Sophia Antipolis, 06904, France.*

Abstract

Opsin photoreceptors are responsible for the absorption of light, transduce information about daily lighting conditions and provide vision. Opsins are also involved in diverse non-visual functions such as the circadian clock. The non-visual opsin 3, also known as encephalopsin, is expressed in skin and its functions remain poorly defined.

The expression of opsin 3 in human skin tissue was evaluated by immunohistochemistry, in keratinocytes and melanocytes by immunodetection. Opsin 3 and β-tubulin colocalization was performed during the keratinocyte mitotic phase. Opsin 3-siRNA transfection was used in primary keratinocytes and successful transfection was confirmed by immunodetection.

These results show that opsin 3 was detected in the epidermis of human skin, both in keratinocytes and melanocytes. Opsin 3 colocalize with β-tubulin during the mitotic phase and participate in keratinocyte cell division. Opsin 3 protein expression was reduced after transfection with opsin 3-siRNA; inhibition of opsin 3-siRNA reduces keratinocyte cell proliferation and promotes the apparition of binucleated cells.

This study highlights an unconventional function of opsin 3 in extra-ocular cells. Opsin 3 appears to be involved in the regulation of late cytokinesis in keratinocytes, and therefore in the maintenance of epidermal homeostasis.

Keywords: *Skin; Keratinocyte; Melanocyte; Photoreceptors; Opsin 3; Mitosis; Cytokinesis.*

Introduction

Humans can see the world in color thanks to photon-sensitive receptors called opsins that are present in the retina. With many millions of photo-sensitive rod and cone cells in the eye, photo transduction is a mechanism activated by a source of light [1]. Many animals utilize light perception to regulate biological processes, such as vision and circadian rhythms [2]. Opsins are seven-transmembrane-helix G protein-coupled receptors (GPCRs) that trap photons with a retinal molecule in the heart of their architecture, thus 'sensing' light [3].

In vertebrates, opsins sense light for visual and non-visual functions, but non-visual roles are less known. The expression of both visual and non-visual opsins has been described in the skin [4], suggesting photosensitivity properties to this tissue. Interestingly, opsin 3, also known as encephalopsin or panopsin, originally identified in the brain [5] is highly expressed in keratinocytes [4]. The encephalopsin name was derived from their strong expression in the brain [6]. Opsin 3 has been suggested to play a role in the entrainment of circadian rhythm and the regulation of pineal melatonin production [7]. Opsin 3 also has been identified as involved in hair growth [8] and skin pigmentation processes [9].

The skin epithelium continually self-renews and can rapidly regenerate after damage. Epidermal homoeostasis is maintained by a fine balance of keratinocyte proliferation and terminal differentiation [10]. The influence of light on skin homeostasis has

previously been described, such as in the pigmentation process, synthesis of vitamin D, endorphins and the neuropeptide called substance P [11; 12; 13; 14]. The cell division process controls the renewal of proliferating epithelial progenitors and hence the extent of production of terminally differentiated lineages. It has been shown that GPCRs are effectors in cell division [15], but the function of GPCRs during mitosis remains poorly understood. GPCRs mostly localize to the plasma membrane, transmit extracellular stimuli into the cytosol and also enter the cytoplasm [15]. In the present work, we investigated the expression of non-visual opsin 3 during the mitosis process in human keratinocytes. Here we report the participation of opsin 3 in the regulation of keratinocyte division. These findings establish an unexpected relationship between sensory GPCRs and mitotic cell divisions.

Materials And Methods

Antibodies and Probes

The following antibodies were used: anti-opsin 3 (LifeSpan BioSciences, Seattle, WA, USA), anti β tubulin (Abcam, Cambridge, UK). Alexa Fluor® coupled secondary antibodies were used (Molecular Probes, Eugene, OR, USA). An Alexa Fluor® 594 Phalloidin probe was used for F-actin detection (A12381, Molecular Probes).

Cell Culture

Normal human epithelial keratinocytes were isolated from skin obtained from healthy female donors who had undergone plastic surgery procedures and given written informed consent (Biopredic International). Keratinocytes were cultured in a keratinocyte serum free medium, supplemented with 50 µg/ml bovine pituitary extract, 5 ng/ml epidermal growth factor (Gibco, Auckland, NZ), and 0.1 mg/ml Primocin™ (Invivogen, San Diego, CA, USA).

RNA Interference

Opsin 3 siRNA (Invitrogen, Carlsbad, CA, USA) was transfected with a final concentration of 50 nM using lipofectamine RNAiMAX (Invitrogen). As a negative control Stealth RNAi Negative Control Duplex (Invitrogen) was used as recommended by the manufacturer.

Immunocyto Fluorescence

Formaldehyde-fixed cells were permeabilized in 0.1% triton X100, then blocked in 1% BSA solution. Cells were incubated with primary antibodies, diluted in phosphate-buffered saline at room temperature, followed by incubation with an Alexa Fluor conjugated secondary antibodies (Invitrogen). Image acquisition was performed using an Axiovert 200M microscope (Carl Zeiss, Oberkochen, Germany). Photos were captured with an EXI blue camera (Qimaging, Surrey, BC, Canada) coupled to Volocity acquisition software (Perkin Elmer, Waltham, MA, USA).

Immunohistological Fluorescence

Normal human epithelial keratinocytes were isolated from skin obtained from healthy female donors who had undergone plastic surgery procedures and given written informed consent (Biopredic International). After removal of subcutaneous fat, tissue was used to obtain 6 mm punch biopsies, which were then fixed in formaldehyde and processed in an automated Shandon Hypercenter XP (Shandon Ltd., Runcor, UK) for paraffin embedding. Sections of 4 µm thickness were cut with a microtome (Shandon) and collected on poly-lysine coated glass slides (Menzel Gläser, Braunschweig, Germany) for immunostaining. Heat-mediated antigen retrieval was performed in citrate buffer before incubation with opsin 3 antibody.

Statistical Analysis

All experiments have been repeated, statistical analyses were performed using JMP software (SAS, Cary, NC, USA). Normality testing of the data was performed with the Shapiro-Wilk test. The one-way analysis of variance (ANOVA) was used to determine whether there was any significant difference between the means of two or more independent groups. Difference between two means was performed with Student's *t*-test. A p-value ≤0.05 was considered statistically significant (*), p-value ≤0.01 as very significant (**) and p-value ≤0.005 as highly significant (***).

Results

Patterns of Opsin 3 Expression in Human Skin

Immunodetection of opsin 3 showed that the protein was broadly detectable in all compartments of the studied tissue. In human adult skin, Opn3 receptor expression was detected in all layers of the epidermis and in many cells located in the dermis (Figure 1).

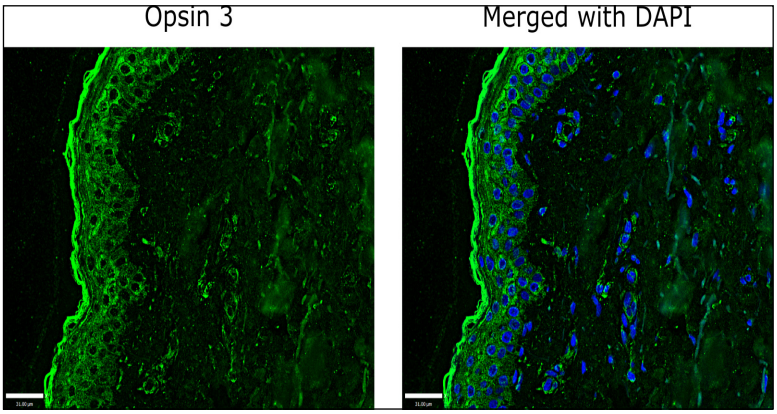

Figure 1. *Immunodetection of opsin 3 receptors in human skin. Representative images of paraffin-embedded sections, using polyclonal antibodies directed against the human opsin 3 receptor (green) and DNA stained with DAPI (blue) respectively (scale bar = 31 µm).*

Patterns of Opsin 3 Expression in Human Keratinocyte and Melanocyte

In cultured keratinocytes, opsin 3 staining showed a cytoplasmic location, with a fluorescence punctate staining (Figure 2A). However, in melanocytes, opsin 3 exhibited a granular staining pattern predominant within the cell body as well as throughout the dendrites (Figure 2B).

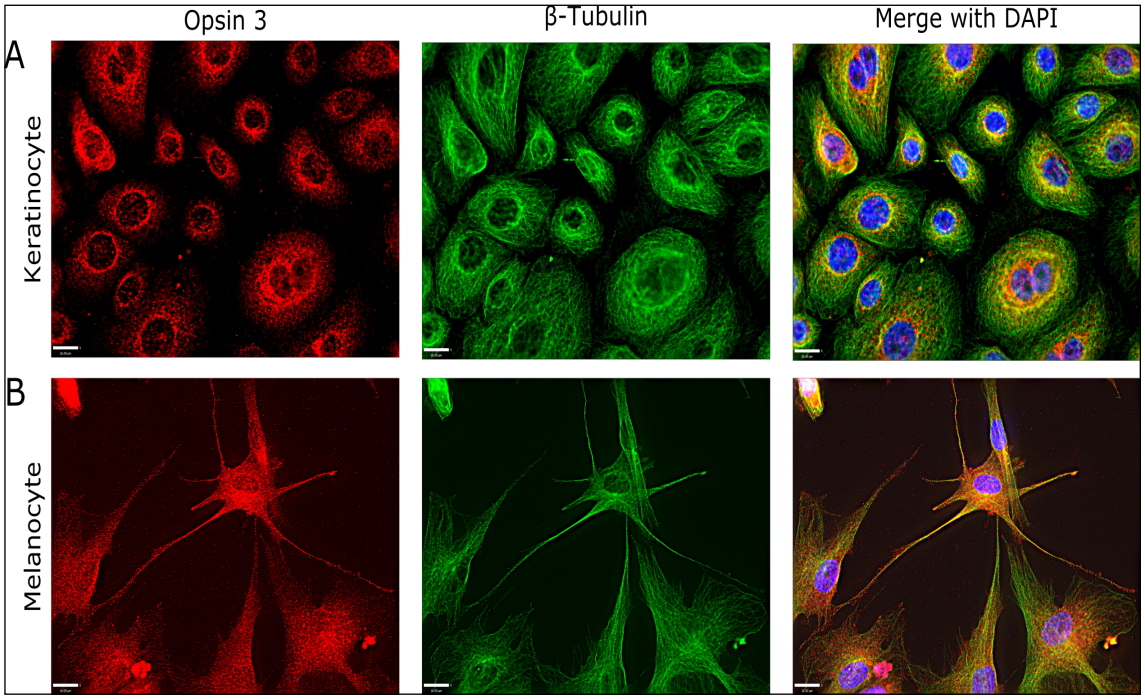

Figure 2. *Immunodetection of opsin 3 receptors in human keratinocytes (A) and melanocytes. (B) Representative images of opsin 3 (red), β tubulin (green) and DNA stained with DAPI (blue) respectively (scale bar = 16 µm).*

Differential Localization of Opsin 3 and Tubulin During Keratinocyte Division

Rather than just being specialized for a sensory event, opsin 3 distribution analysis during cell division revealed a different pattern of localization (Figure3) that could be observed in interphase cells (Figure2A). During the mitosis step corresponding from prometaphase to anaphase, the opsin 3 localized to different areas related to the β tubulin network, such as the mitotic pole (Figure 2). The opsin 3 staining during cytokinesis and late cytokinesis showed a localization at the cleavage furrow, again in relation with the β tubulin staining.

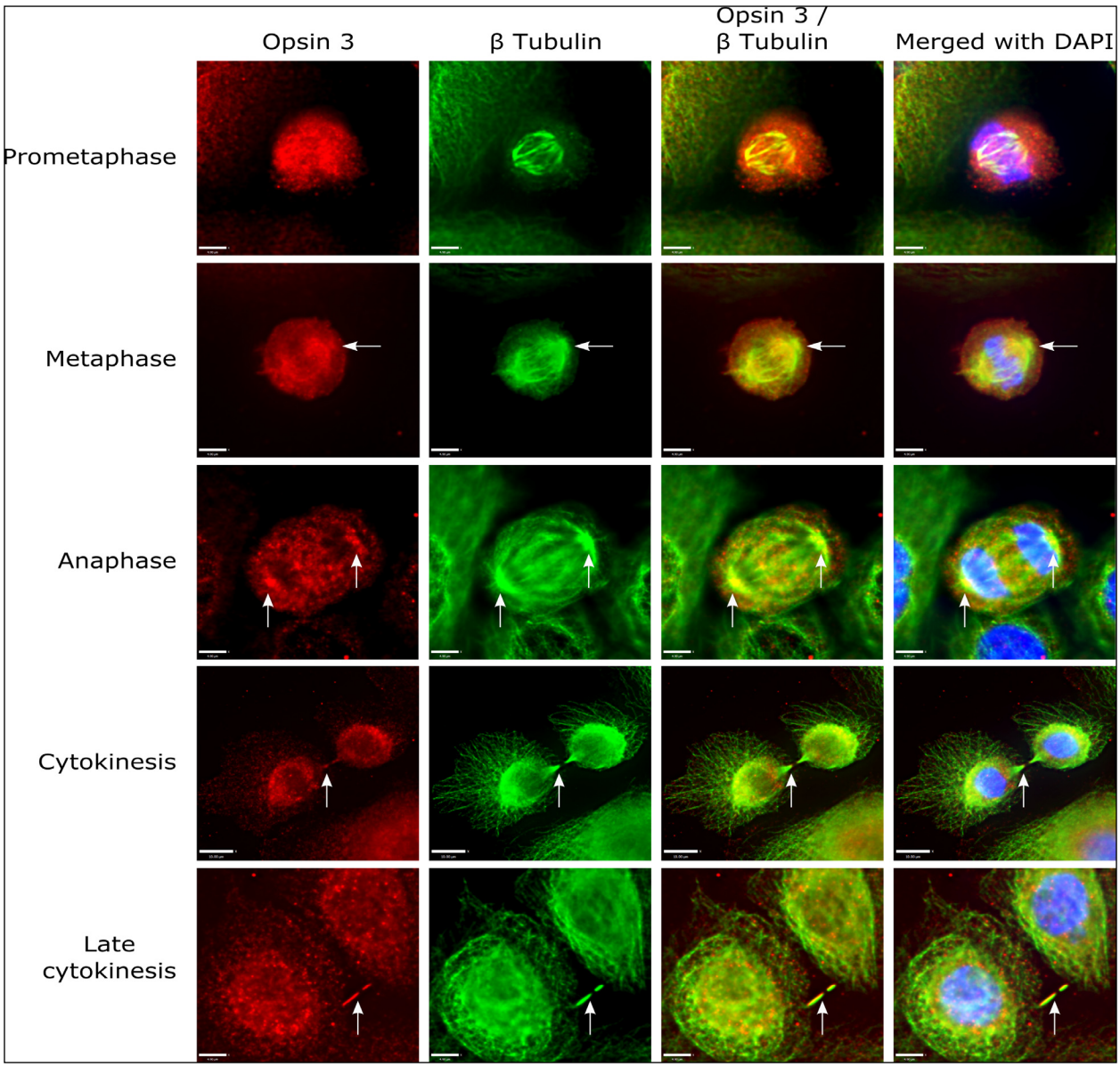

Figure 3. *Immunodetection of opsin 3 receptors in mitotic human keratinocytes. Representative images of opsin 3 (red), β tubulin (green) and DNA stained with DAPI (blue), during prometaphase, metaphase, anaphase, cytokinesis and late cytokinesis. White arrows show colocalization between opsin 3 and β-tubulin at the mitotic pole and the cleavage furrow (scale bar = 4.90 µm and scale bar = 10 µm for the cytokinesis phase pictures).*

Interference with Opsin 3 Function Reduces Keratinocyte Proliferation

To better understand the effects of opsin 3 inhibition on mitosis, we used small interfering RNA (siRNA) to deplete endogenous opsin 3 in keratinocytes. Surprisingly, the opsin 3 depletion resulted in a drastic reduction of cell proliferation, and the appearance of large and more multinucleated cells through nuclear division without cytokinesis (Figure 4). Generally, a larger-cell subpopulation of keratinocytes represents more terminally differentiated keratinocytes. Opsin 3 siRNA resulted in multinucleated cells with a single cytoplasmic compartment, 48 hours after transfection. The rate of multi-nucleated cells was observed by immunodetection and quantified on a representative sample. In our results the negative control siRNA condition showed 2.6% of keratinocytes were multinucleated cell (n=319 cells; SEM=0.5%), and the opsin 3 siRNA condition showed 10.9% of keratinocytes were multinucleated (significant * with Student's t test n=307 cells; SEM=2.7%).

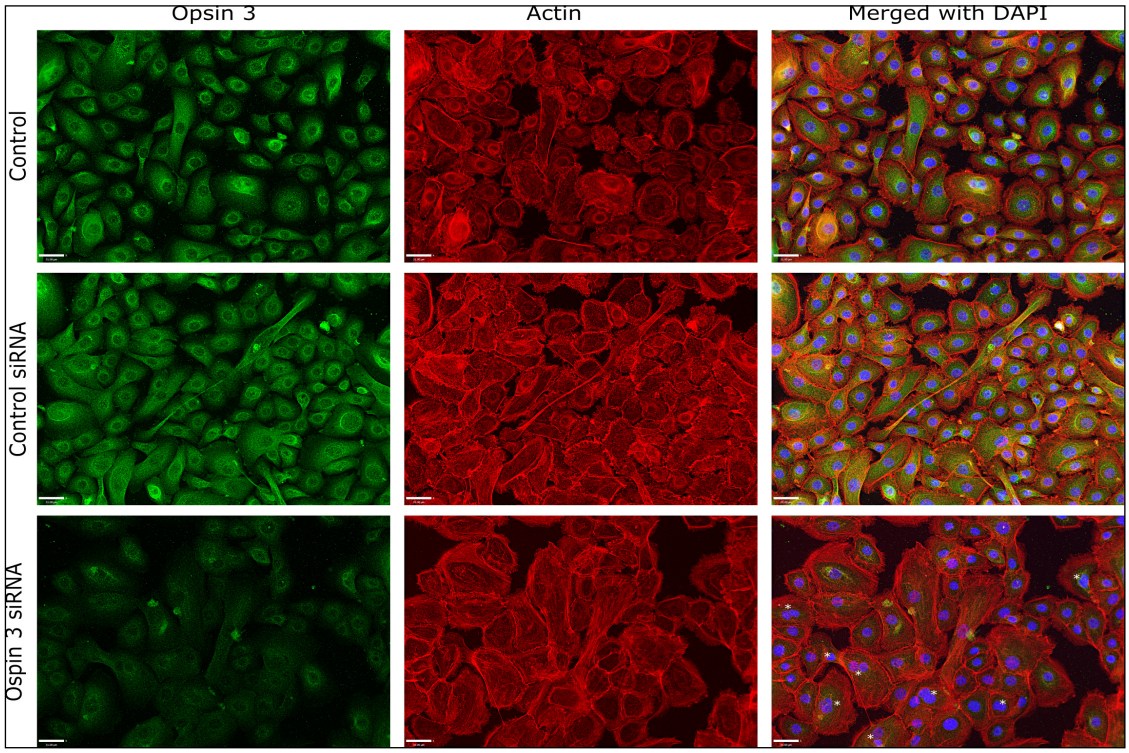

Figure 4. *siRNA mediated inhibition of opsin 3 in human keratinocytes. Representative images of opsin 3 (green), actin (red) and DNA stained with DAPI (blue) 48 hours after transfection. White stars show multinucleated cells (scale bar = 31 µm).*

Discussion

Several kinds of endogenous animal opsins have been isolated and characterized; they are phylogenetically and functionally classified into eight groups [16; 17; 18]. Vertebrate opsin 3 was suggested to form blue-sensitive pigments with the ability to activate G proteins [19]. The skin is the interface between the body and the external environment and acts primarily as a physical barrier, while also controlling evaporation and regulating body temperature. The skin is also a very complex community of cells that handles many functions such as immune defense, as well as the perception and transmission of sensation [20]. The existence of an organized system of photoreceptors in the skin becomes more and more evident with the number of opsin receptors that are described in skin cells such as keratinocytes and melanocytes [4, 21, 22, 23, 24].

In this study, we succeeded in obtaining the expression patterns of human opsin 3 in skin and observed that opsin 3 exhibits a broad tissue distribution. mRNAs of opsin 3 are found in keratinocytes and in melanocytes [4]. We therefore further examined the opsin 3 localization in these "non-visual cells" by using primary cultures from adult skin. In melanocytes, opsin 3 had a punctate granular distribution in the cytoplasm in both cell types. In melanocytes, we showed that opsin 3 accumulation is enriched in a very granular pattern, along dendrites and dendritic tips. This pattern is suggestive of melanosomal localization, but this still needs to be confirmed by colocalization with Tyrosinase and Pmel17. In keratinocytes, immunoreactivity was also observed as granular structures in interphase cells. Our observation in mitotic keratinocytes, of the subcellular localization of opsin 3 away from the plasma membrane for G proteins, likely contributes to their function in cell division.

GPCRs modulate diverse physiological and behavioral signaling pathways by way of changes in receptor activation and inactivation states. Our findings are intriguing in light of earlier studies that showed that the G-coupled receptor participated in mitosis [15]. Cell division is a highly coordinated process that involves the relay of signals from both the outside and inside of the cell. Opsin 3 is a potential candidate to relay a signal from the outside, as the skin can be exposed directly to both natural light and artificial blue light, which can emanate from sources such as LED electronic devices.

Conclusion

Understanding how cells respond to extracellular cues is less explored, and the implication of the presence of few sensory receptors during mitosis have already been reported, including OPN1MW (opsin1, medium-wave-sensitive), OR2A4 (olfactory receptor family 2, subfamily A member 4) and TAS2R13 (taste receptor, type2, member 13) [15]. To complete the cell cycle, the cleavage furrow draws the plasma membrane toward the cell center, pinching the cytoplasm into two lobes that are subsequently separated into two cells. Throughout a human's life, the epidermis must constantly rejuvenate and replace dying cells with fresh ones. Therefore, opsin 3 appears important for cleavage furrow formation and thus its participation in keratinocyte renewal is required for maintaining epidermis homeostasis.

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