Does Blue Light Restore Human Epidermal Barrier Function via Activation of Opsin During Cutaneous Wound Healing?

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Background and Objective: Visible light has beneficial effects on cutaneous wound healing, but the role of potential photoreceptors in human skin is unknown. In addition, inconsistency in the parameters of blue and red light-based therapies for skin conditions makes interpretation difficult. Red light can activate cytochrome c oxidase and has been proposed as a wound healing therapy. UV-blue light can activate Opsin 1-SW, Opsin 2, Opsin 3, Opsin 4, and Opsin 5 receptors, triggering biological responses, but their role in human skin physiology is unclear.

Materials and Methods: Localization of Opsins was analyzed *in situ* in human skin derived from face and abdomen by immunohistochemistry. An *ex vivo* human skin wound healing model was established and expression of Opsins confirmed by immunohistochemistry. The rate of wound closure was quantitated after irradiation with blue and red light and mRNA was extracted from the regenerating epithelial tongue by laser micro-dissection to detect changes in *Opsin 3* (*OPN3)* expression. Retention of the expression of Opsins in primary cultures of human epidermal keratinocytes and dermal fibroblasts was confirmed by qRT-PCR and immunocytochemistry. Modulation of metabolic activity by visible light was studied. Furthermore, migration in a scratch-wound assay, DNA synthesis and differentiation of epidermal keratinocytes was established following irradiation with blue light. A role for OPN3 in keratinocytes was investigated by gene silencing.

Results: Opsin receptors (OPN1-SW, 3 and 5) were similarly localized in the epidermis of human facial and abdominal skin *in situ*. Corresponding expression was confirmed in the regenerating epithelial tongue of *ex vivo* wounds after 2 days in culture, and irradiation with blue light stimulated wound closure, with a corresponding increase in *OPN3* expression. Expression of Opsins was retained in primary cultures of epidermal keratinocytes and dermal fibroblasts. Both blue and red light stimulated the metabolic activity of cultured keratinocytes. Low levels of blue light reduced DNA synthesis and stimulated differentiation of keratinocytes. While low levels of blue light did not alter keratinocyte migration in a scratch wound assay, higher levels inhibited migration. Gene silencing of *OPN3* in keratinocytes was effective (87% reduction). The rate of DNA synthesis in OPN3 knockdown keratinocytes did not change following irradiation with blue light, however, the level of differentiation was decreased.

Conclusions: Opsins are expressed in the epidermis and dermis of human skin and in the newly regenerating epidermis following wounding. An increase in OPN3 expression in the epithelial tongue may be a potential mechanism for the stimulation of wound closure by blue light. Since keratinocytes and fibroblasts retain their expression of Opsins in culture, they provide a good model to investigate the mechanism of blue light in wound healing responses. Knockdown of OPN3 led to a reduction in early differentiation of keratinocytes following irradiation with blue light, suggesting OPN3 is required for restoration of the barrier function. Understanding the function and relationship of different photoreceptors and their response to specific light parameters will lead to the development of reliable light-based therapies for

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Conflict of Interest Disclosures: All authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest and have disclosed the following: Dr. Castellano-Pellicena American Society for Laser Surgery & Medicine; award "Best of photobiomodulation session" during the ASLMS conference 2017. This study was conducted under the European Marie-Curie Actions Programme, Grant agreement no. 607886, Irene Castellano Pellicena and Charles Mignon were Early Stage Researchers and Natallia E. Uzunbajakava, Vladimir A. Botchkarev and M. Julie Thornton were the members of a scientific supervisory team. http://www.skinlight-classic.eu/about_classic/.

Contract grant sponsor: European Commission 7th Framework Programme for Research and Technical Development - Marie Curie Innovative Training Networks (ITN); Contract grant number: 607886.

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Accepted 6 August 2018
Published online in Wiley Online Library (wileyonlinelibrary.com).
DOI 10.1002/lsm.23015

cutaneous wound healing. Lasers Surg. Med. © 2018 Wiley Periodicals, Inc.

Key words: photobiomodulation; blue light; red light; Opsins; OPN1-SW; OPN3; OPN5; epidermal keratinocytes; *ex vivo* wound healing and epidermal barrier

INTRODUCTION

Photobiomodulation (PBM) as a clinical application for wound healing is impaired by a lack of complete understanding of all involved molecular targets [1], which include cytochrome c oxidase (CCO) [2], nitrozated proteins [3,4], flavoproteins generating reactive oxygen species (ROS) [5–7] and light-activated calcium channels [8]. Previously it was reported that interaction of CCO with red light [2] induces a shift in the redox potential of the cell, inducing transcriptional changes modulating proliferation, metabolism and motility, all important in wound healing [9].

Currently, there is growing interest in the Opsins (OPNs) which are G-protein coupled receptors (GPCRs), as potential targets for light therapy. In humans, the visual OPNs are OPN1 and OPN2 (rhodopsin) which are the main photoreceptors in the eye [10]. Human OPN1 is further subdivided into three forms, OPN1-SW (short wavelength), OPN1-MW (medium wavelength), and OPN1-LW (long wavelength) which are found in cones while OPN2 is found in rods. The maximum absorption spectrum for OPN1-SW is in the violet–blue range (420–440 nm), while OPN2 is activated by cyan light (496 nm) in the eye [11]. Furthermore, there is increasing evidence that OPN2 in the skin is activated by UVA-violet light in humans [12,13] and mice [14]. There are also non-visual OPNs, which are OPN3 (encephalopsin), OPN4 (melanopsin), and OPN5 (neuropsin) with absorption bands that extend over the UV-A-visible spectrum with corresponding peaks at 460 nm [15], 496 nm [16], and 380 nm [17].

In general, red light (600–760 nm) was reported to stimulate proliferation and migration of human epidermal stem cells [18] and keratinocytes [19], increase human fibroblast numbers from normal skin and hypertrophic scars [20], and encourage migration of fibroblasts from patients with diabetes [21]. This suggests beneficial roles for red light therapy in re-epithelialization and extracellular matrix formation during wound healing.

In contrast, blue light (450–490 nm) was reported to inhibit proliferation of neonatal foreskin keratinocytes at 33 J/cm^2 with onset of apoptosis at higher doses (100 J/cm^2) [3]. Blue light was also reported to induce early differentiation of keratinocytes at low doses (5 J/cm^2), while higher settings (66 and 100 J/cm^2) induced late differentiation [3]. Reduced proliferation and metabolic activity has also been observed in primary cultures of human dermal fibroblasts after irradiation with blue light [6,22]. Recently, Mignon et al. demonstrated a stimulatory effect with a single treatment of 450 nm light at 2 J/cm^2 on metabolic activity of dermal fibroblasts, with suppressive action following additional irradiations [23]. In a scratch-wound assay, high-dose blue light (55 J/cm^2) inhibited fibroblast migration, while lower doses (2, 5, and 10 J/cm^2) had no effect [24].

In wound healing, red light is thought to stimulate proliferation and re-epithelialization, while blue light has an antimicrobial effect to combat infection [25,26]. The anti-proliferative effect is the basis for the use of blue light therapy for psoriasis treatment [27–29], while its modulation of dermal fibroblasts has potential for the treatment of hypertrophic scarring in the latter stages of wound healing [30]. Finally, blue light is also able to induce vasodilatation in mice *via* activation of OPN4, a very important function to ensure the blood flow reaches the wound site, especially during chronic wound healing [31]. Yet, the role of blue light in other stages of wound healing has not been investigated. Here, we suggest a role for low-dose blue light in the early differentiation of keratinocytes to restore the barrier function of the epidermis.

After the discovery of OPN2 expression in murine melanocytes in culture [32], Tutsumi et al. were the first to identify the presence of visual OPNs (OPN1-SW and M/LW and OPN2) in human facial skin. In humans, there is a growing evidence for photosensitivity of OPNs outside of the image-forming visual system [12,13,15,17,33–35]. Since most Opsins have their absorption peak in the short wavelength region of the optical spectrum, blue, violet, and UV-A light will be the most effective at activating OPN-mediated signaling pathways. In human melanocytes, OPN2 was reported to mediate photon reception and signal transduction following UV-A exposure, leading to calcium mobilization and melanogenesis [13]. More specifically, OPN2 mRNA was up-regulated by violet light (410 nm) in cultured neonatal foreskin keratinocytes [12]. The non-visual OPN3, a photoreceptor generally found in animals such as the pufferfish [15], has recently been identified in human hair follicles, including the stem cells [33], and it was activated by low-dose blue light to increased proliferation of cultured outer root sheath keratinocytes [33]. Furthermore, OPN4 was responsible for the vasodilatation response to blue light in mice [31]. Collectively, these findings highlight a function for OPNs in human skin, opening new fields of opportunities for developing light-based therapies specifically targeting OPNs.

The role of OPNs in wound healing has never been addressed. Therefore, the first aim of this study was to investigate the expression and localization of OPNs in human skin from different anatomical regions, and in the regenerating epithelial tongue of an *ex vivo* human skin wound-healing model. Then, to establish whether blue or red light could stimulate wound closure in an *ex vivo* wound-healing model, and whether this could be related to expression of OPN3 in the regenerating epidermis. The second aim was to investigate the retention of OPNs in primary epidermal keratinocytes and dermal fibroblasts in culture, and then, to study whether blue or red light can alter functional responses of epidermal keratinocytes important in wound healing, that is, metabolic activity, proliferation, differentiation and migration and if knockdown of the OPN3 gene would affect light-triggered cell behavior.

MATERIALS AND METHODS

Expression of Opsins in Human Skin

Female human skin was obtained with full written consent adhering to the Declaration of Helsinki principles and under human tissue act guidelines. Facelift or abdominoplasty procedures were always performed in the morning and skin processed in the afternoon of the same day. Seven micrometer cryosections ($n = 4$ donors, 44–63 years) were fixed in acetone before blocking with 5% bovine serum albumin (BSA) and 5% donkey serum (DKS) in phosphate-buffered saline (PBS) for 1 hour. Primary antibodies were diluted in 1% BSA and 1% DKS and incubated overnight at 4°C; 1:200 OPN1-SW (AB5407, Millipore, Amsterdam-Zuidoost, the Netherlands), 1:100 OPN3 (ab66742 for immunohistochemistry (IHC) and ab75285 for immunocytochemistry (ICC), Abcam, Cambridge, UK), 1:200 OPN5 for IHC and 1:500 for ICC (ab199668, Abcam). A negative control (omission of primary antibody) was included in the experimental procedure. Double staining was performed with 1:200 KRT14 (ab51054 or ab7800, Abcam). Incubation with 1:200 Alexa-488 (ab150073, Abcam) and Alexa-647 (ab150115, Abcam) was for 1 hour at 37°C. Slides were mounted using VECTASHIELD® containing DAPI (VECTOR). Images were taken using a confocal microscope (Leica Microsystems B.V., Amsterdam, the Netherlands).

Light-Emitting Devices

The effect of light was investigated using proprietary LED-based devices, where the irradiance, beam homogeneity, temperature of culture plates, and prevention of light leakage between the plates was carefully controlled or monitored [3,23,33,36]. The temperature of the wells was measured with a FLIR A655sc Infrared Camera, (FLIR® Systems, Inc, Meer, Belgium) at the start of the treatment, which was approximately 37°C, and at the end, when the temperature had gone down to 24°C.

The impact of selected discreet wavelengths across visible to near-infrared spectrum on metabolic activity of keratinocytes *in vitro* was investigated using an LED-based device emitting 447, 505, 530, 655, and 850 nm wavelengths and housing 24 well-plates [23]. Response of an *ex vivo* wounded skin model to light was studied using LED-based devices emitting a single wavelength (either 453 or 656 nm) [3,33,36]. The 453 nm LED-based device was also used to study migration, DNA synthesis and differentiation of keratinocytes. Media was refreshed after every light treatment.

Effect of light on an ex vivo human skin wound healing model. An *ex vivo* wound healing assay was established [37]. Two parallel incisions were made using an in-house double blade scalpel (1 mm wide). Rectangular wounds (60–80 mm^2) surrounded by epidermis at both sides were cultured at the air liquid interface (Netwell inserts, Corning,) in phenol red-free DMEM with 10% foetal bovine serum [38], 250 U/ml penicillin and 250 μg/ml streptomycin (Pen/Strep), 7.5 μg/ml of amphotericin B and 5% glutaMAX. *Ex vivo* wounds were irradiated daily with two proprietary LED-devices emitting 453 nm light [3,33,36] at 2 J/cm^2 or 656 nm at 30 J/cm^2. Images were taken using a wide-field microscope (Leica) and wound area was measured using Image J software. Samples were collected at day 0 (immediately after wounding) and day 2 (4 hours after light treatment) for IHC of OPNs (see above) and KRT17 (1:200, ab109725, Abcam).

Laser micro-dissection of regenerating epidermis. Cryosections from *ex vivo* wounds were collected onto membrane slides (Molecular Machines and Industries [MMI]) and stained with the H&E RNase free kit (MMI). The epithelial tongue was collected into a 0.5 ml diffuser cap collection tube (MMI) with a Nikon T2000 laser capture microscope. RNA isolation was performed using RNeasy Micro Kit (QIAGEN) and RNA amplification with the Arcturus™ RiboAmp® PLUS Kit (ThermoFisher Scientific, Carlsbad, CA). RNA quality was confirmed with Agilent 2100 bioanalyzer (Agilent, Santa Clara, CA).

Quantitative real-time PCR (qRT-PCR) [39] was performed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, UK) and SYBR Green Supermix (Applied Biosystems); OPN primers were used at 1 μM (Table 1). Level of expression (Ct ($C_t^{\Delta\Delta}$) equitation method) was analyzed with respect to the GAPDH housekeeping gene. Human universal reference total RNA (Clontech) was the positive control.

Cell culture of human primary epidermal keratinocytes and dermal fibroblasts. Primary cultures of human epidermal keratinocytes and dermal fibroblasts from healthy females were established [40]. Keratinocytes (passage 2–5) were cultured in serum free supplemented keratinocyte growth medium 2 (Promocell, UK) and Pen/Strep. Media was changed to EGF-free and phenol red-free starvation media 24 hours before each experiment. Papillary fibroblasts (passage 3–8) were cultured in DMEM

TABLE 1. Primer Sequences of OPN1-SW, OPN3, OPN5, and KRT10 for qRT-PCR

Accession number	Gene	Primer sequence (sense/antisense)	T_{ann}
NM_001708	OPN1-SW	5′CGCCAGCTGTAACGGATACT3′/5′CCGAAGGGCTTACAGATGAC3′	63.1°C
NM_014322	OPN3	5′CAATCCAGTGATTTATGTCTTCATGATCAGAAAG3′/ 5′GCATTTCACTTCCAGCTGCTGGTAGGT3′	63.1°C
NM_181744	OPN5	5′CTAGACGAAAGAAGAAGAAGCTGAGACC3′/5′GCGGTGACAAAAGCAAGAGA3′	63.1°C
NM_000421.3	KRT10	5′AGCATGGCAACTCACATCAG3′/5′TGTCGATCTGAAGCAGGATG3′	62.4°C

T_{ann}, annealing temperature.

supplemented with 10% FBS, Pen/Strep, 7.5 μg/ml of amphotericin B and 5% glutaMAX. Media was changed to 2% FBS and phenol red-free starvation media 24 hours before each experiment.

Total RNA from keratinocytes (facial skin, $n = 4$) and fibroblasts (breast, face, abdomen and scalp skin, $n = 8$) was isolated and qRT-PCR of *OPN1-SW*, *OPN2*, *OPN3*, and *OPN5* performed as previously described. RT-PCR of OPN4 from fibroblast RNA only was performed as previously described [41]. For ICC, cells were cultured in Millicell EZ 8-well glass slides (Merck, UK).

Effect of light on the metabolic activity of keratinocytes. Keratinocytes in 24 well-plates with a black frame (Porvair Science, Wrexham, UK) were irradiated with blue (447 nm, 50 mW/cm^2, 2 J/cm^2), cyan (505 nm, 30 mW/cm^2, 2 J/cm^2), green (530 nm, 30 mW/cm^2, 2 J/cm^2), red (655 nm, 40 mW/cm^2, 30 J/cm^2) or near infrared (NIR) (850 nm, 40 mW/cm^2, 60 J/cm^2) light using the proprietary LED-based devices [23]. Alamar Blue® (Thermo Fisher Scientific) assay was performed after 24 hours [23] and relative metabolic activity analyzed with respect to corresponding non-irradiated cultures.

Effect of blue light on the migration of keratinocytes. Keratinocytes in 12-well plates were scratched and the proprietary LED-based device, (453 nm, 50 mW/cm^2, 2 J/cm^2) was used to irradiate keratinocytes. Images at 0, 12, and 21 hours after scratching were analyzed using Image J.

Effect of OPN3 silencing and blue light on DNA synthesis and differentiation of keratinocytes. Keratinocytes cultured on 0.5% gelatin coated coverslips were synchronized [42] before transfection with complexes of lipofectamine RNAiMax (Invitrogen, Bleiswijk, the Netherlands) and smartpool siRNA (50 nM) directed against OPN3 (siOPN3) or non-targeting siRNA control (NTRC) (ON-Targetplus, Dharmacon). After 24 hours, cells were irradiated with 453 nm (50 mW/cm^2, 2 J/cm^2) light and the thymidine analogue EdU (10 μM), (Click-iT® EdU Imaging Kits, Invitrogen) was added. After 10 hours cells were fixed with 4% paraformaldehyde (PFA) for double staining of EdU fluorescence (green) and KRT10 expression (red) by ICC (ab76318, Abcam). Images were taken using the slide scanner, Pannoramic MIDI II (3DHISTECH) and Image J was used to quantify the relative percentage of EdU and KRT10 positive cells. Transfection efficiency and *KRT10* expression was performed by qRT-PCR (Table 1).

RESULTS

Opsins are Similarly Expressed in Human Facial and Abdominal Skin

OPN1-SW, OPN3, and OPN5, were all expressed in female facial and abdominal skin (Fig. 1), while OPN2 has previously been shown to be expressed in facial skin *in situ* [43]. OPN1-SW and OPN3 were expressed throughout the epidermis, with OPN1-SW mainly in the stratum granulosum, while OPN3 was more prominent in the basal layer (where the basal keratinocytes were identified using KRT14). OPN1SW and OPN3 were also expressed in the dermis. In contrast, the expression of OPN5 was confined exclusively to the basal keratinocytes.

An Ex Vivo Human Skin Wound Healing Model Regenerates New Epithelium With an Induced Expression of KRT17

An *ex vivo* human skin wound healing model was established. After two days in culture, the wound edges visibly started to close (Fig. 2) and histology confirmed the formation of a new epithelial tongue from the wound edges. This was further confirmed by the high expression of KRT17 at the wound margins and in the migrating epithelial tongue after 2 days in culture, in comparison to skin biopsies taken immediately after wounding (Fig. 2F and E).

Opsins are Expressed in the Epithelial Tongue of Human Skin During Wound Repair *Ex Vivo*, and Light Accelerates Wound Closure

OPN expression was also demonstrated in the regenerating epithelium of human *ex vivo* wounds after 2 days in culture. KRT14 was expressed in the new epithelial tongue (Fig. 3). OPN1SW was co-localized with KRT14 in the apical epithelial tongue, while both OPN3 and OPN5 co-localized with KRT14 in the basal epithelial tongue of the regenerating epidermis.

To establish whether light modulates expression of OPNs during wound healing, the epithelial tongue was isolated by laser micro-dissection from non-irradiated biopsies after blue (2 J/cm^2) or red (30 J/cm^2) light exposure. Low-level blue light (453 nm; 2 J/cm^2) induced *OPN3* expression, while red light had no effect.

The speed of closure for each wound was analyzed at day 2 following light treatment. While it was variable between donors, intra-donor response was consistent. Low dose of blue light (2 J/cm^2) increased the rate of closure in Donor 2 and 3, while red light (30 J/cm^2) increased wound closure only in Donor 2 (Fig. 3).

Primary Epidermal Keratinocytes and Dermal Fibroblasts Derived From Human Skin Retain Expression of Opsins in Culture

Both mRNA and protein expression of OPNs was confirmed in primary human keratinocytes and papillary dermal fibroblasts. Keratinocytes and fibroblasts expressed mRNA and protein for OPN1SW, which was confined to the peri-nuclear region in both cell types (Fig. 4). Likewise, OPN3 mRNA and protein was also expressed in keratinocytes and fibroblasts, although protein expression was diffusely localized in the cytoplasm and cell membrane. Comparison of the relative mRNA expression of *OPN3* showed significantly higher levels in keratinocytes compared to fibroblasts. In contrast, *OPN5* (both mRNA and protein) was only expressed in keratinocytes. Interestingly, expression of OPN2 was not retained in keratinocytes in culture while it was found in dermal fibroblasts at both mRNA and protein level (data not shown). *OPN4* mRNA was not detected in dermal

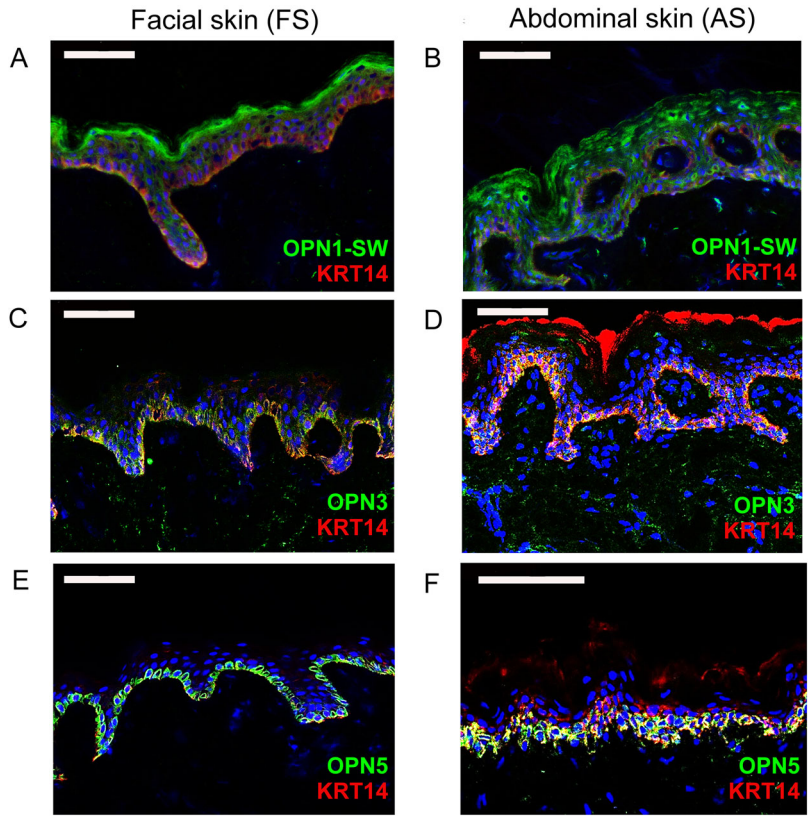

Fig. 1. Localization of OPN1-SW, OPN3 and OPN5 in facial and abdominal human skin by immunohistochemistry. (**A**) Localization of OPN1-SW (green) and KRT14 (red) in facial skin (FS). (**B**) Localization of OPN1-SW (green) and KRT14 (red) in abdominal skin (AS). (**C**) Localization of OPN3 (green) and KRT14 (red) in FS. (**D**) Localization of OPN3 (green) and KRT14 (red) in AS. (**E**) Localization of OPN5 (green) and KRT14 (red) in FS. (**F**) Localization of OPN3 (green) and KRT14 (red) in AS. Yellow denotes co-localization. Nuclei are counterstained with DAPI (blue). Scale bars = 75 μM. Representative images from four individual donors. Negative control (omission of primary antibody) was included to confirm there was no non-specific binding (data not shown).

fibroblasts in culture so further analysis was not carried out (data not shown).

Light Has a Physiological Effect on Epidermal Keratinocyte Function in Culture

To establish a physiological effect of light on human keratinocytes, cultures were irradiated with 2 and 30 J/cm^2 of blue, cyan and green light (447, 505, and 530 nm), as well as 30 J/cm^2 of red light (655 nm) or 60 J/cm^2 of NIR light (850 nm) and metabolic activity was quantitated. A statistically significant increase in metabolic activity was stimulated by 2 J/cm^2 of blue, cyan and red light (Fig. 5). However, 2 J/cm^2 of green light, 30 J/cm^2 of blue, cyan and green light and 60 J/cm^2 of NIR had no effect.

Since OPN1-SW and OPN3 have an absorption peak in the blue light wavelength, to further understand their potential role on epidermal keratinocytes, the effect of 2 J/cm^2 of 453 nm (blue) light on cell morphology, cell differentiation, DNA synthesis and cell migration in a scratch-wound assay was investigated. Cultures of human primary epidermal keratinocytes, following irradiation with 2 J/cm^2 of blue light, showed no changes in morphology and colony formation ability after treatment (Fig. 5). DNA synthesis of keratinocytes was reduced following exposure to low levels of blue light, while differentiation, as shown by expression of KRT10, a marker of early differentiation, was induced (Fig. 5). In contrast, blue light had no effect on keratinocyte migration in a scratch-wound assay. To further investigate the effect of blue light on their migratory ability, keratinocytes were exposed to a higher dose (30 J/cm^2) in a scratch-wound assay, which reduced migration as early as 12 hours.

Blue Light-Induced Differentiation of Cultured Epidermal Keratinocytes Is Regulated by OPN3

Since low-irradiance blue light decreased DNA synthesis and increased differentiation (Fig. 5), the effect of blue light following knockdown of OPN3 was evaluated. The efficiency of OPN3 knockdown was confirmed by an 87% reduction of *OPN3* mRNA in siOPN3 keratinocytes compared to control NTRC (Fig. 6). The reduction in OPN3 protein expression was confirmed by Western Blot

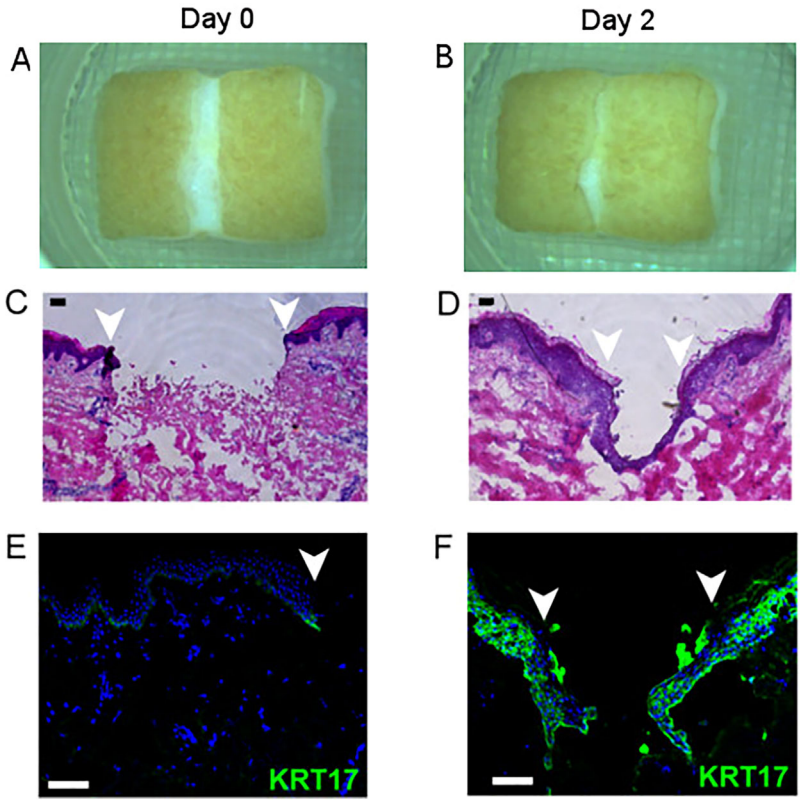

Fig. 2. Human *ex vivo* wound healing model. (**A**) Representative image of *ex vivo* human skin immediately after wounding and (**B**) two days after wounding and cultured in the air liquid interphase ($n = 3$ donors). (**C**) Hematoxylin and eosin staining *ex vivo* human skin immediately after wounding showing removal of epidermis and upper dermis. (**D**) Hematoxylin and eosin staining of *ex vivo* wounds after two days in culture. Arrows denote wound edge. (**E**) KRT17 (green) expression in human skin immediately after wounding. (**F**) KRT17 (green) expression in *ex vivo* wounds after two days in culture. Nuclei are counterstained with DAPI (blue). Scale bar = 75 μM.

48h after silencing demonstrating it had decreased by 61.42% (data not shown). The expression of *KRT10* mRNA did not change, confirming OPN3 knockdown had no effect on keratinocyte differentiation.

Following knockdown of OPN3, keratinocytes irradiated with 2 J/cm^2 of blue light significantly reduced EdU incorporation, a marker of DNA synthesis. However, DNA synthesis was similarly reduced in NTRC keratinocytes by blue light. In contrast, while blue light induced differentiation in NTRC keratinocytes, this effect was abrogated in OPN3 knockdown keratinocytes (Fig. 6).

DISCUSSION AND CONCLUSIONS

Since the first sequence of an Opsin, bovine rhodopsin, was determined by protein sequencing in 1982, more than 1,000 opsins have been identified, 9 of them are present in the human genome [10].

Beyond vision, the OPN family have been implicated in other functions. For example, OPN4 is considered to be a circadian clock photoentrainment molecule in mammals [44–47], while OPNs have also been implicated in functions related to temperature regulation [48–50]. Fascinatingly, following the work of Miyashita et al. describing the expression of OPN2 and cone opsins in murine melanocytes [32], the presence of visual opsins, OPN1 and OPN2 that mediate day-light (color) and twilight vision in the human eye [51] has been reported in human facial epidermis [43], suggesting a potential light-sensitive role in the skin. Indeed, since then, the biological role of OPNs in light photoreception in skin is emerging as a new, exciting field in PBM [12,13,33–35], as well as in other tissues beyond the eye [15,17,31,52,53].

Interestingly, we have observed the presence of OPN1-SW, which is activated by violet-blue light (420–440 nm) [10], a wavelength that does not penetrate very far into the skin [54], not only in the superficial layers of the epidermis in agreement with Tutsumi et al., but strikingly also in the papillary dermis. In contrast, the expression of OPN3 (with an absorption peak at a longer wavelength of 460 nm) was mainly confined to the stratum basale, albeit lower expression was also seen in the suprabasal layer. This is partially in agreement with localization of OPN3 in human scalp [33], where expression was higher in differentiated layers of the epidermis compared to facial or abdominal skin in this study. The expression of OPN3 in basal keratinocytes may be explained by its absorption at a slightly longer wavelength (465 nm) [15], compared to OPN1-SW [10]. However, we

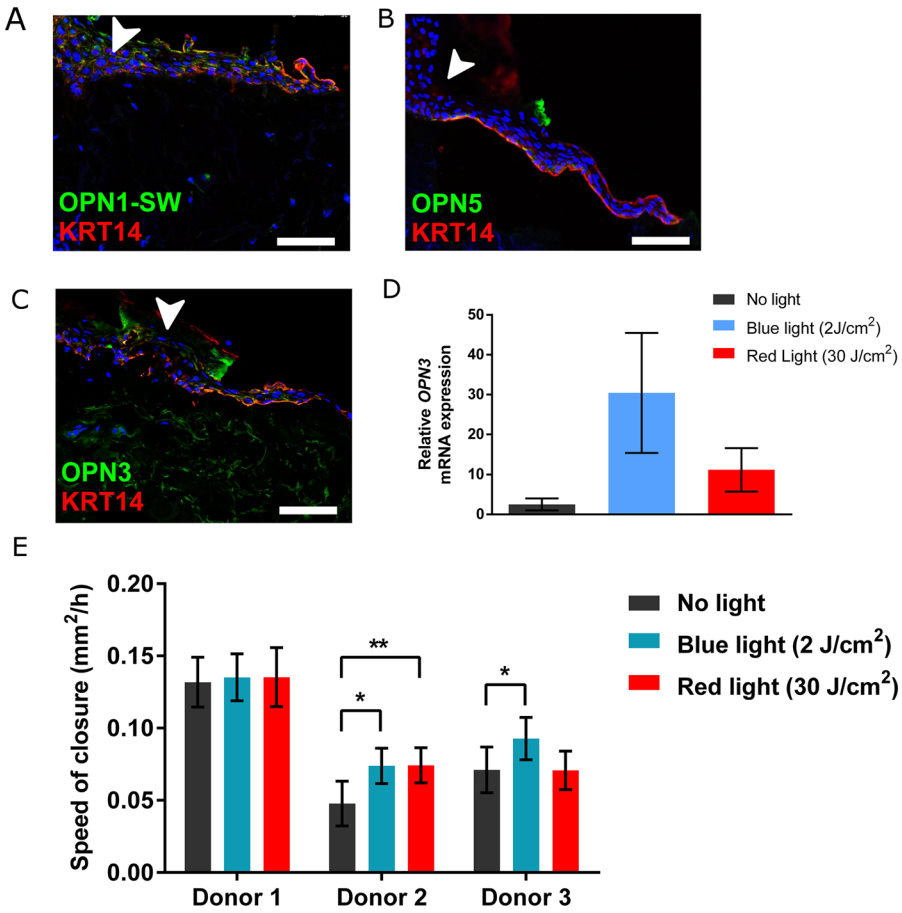

Fig. 3. Expression of OPNs and effect of blue and red light during re-epithelialization. (**A–C**) Localization of KRT14 (red) and OPN (green); OPN1-SW (**A**), OPN5 (**B**) and OPN3 (**C**) in the newly formed epithelial tongue of *ex vivo* wounds after two days in culture. Arrows denote growth of new epidermis. Nuclei are counterstaining with DAPI. Scale bar = 75 μM. Representative images from 4 individual donors. (**D**) Relative mRNA expression of OPN3 in the newly formed epithelial tongue after irradiation with blue light (2 J/cm^2) or red light (30 J/cm^2). Data was calculated relative to GAPDH and presented as the mean of the normalized relative expression of $n = 2$–3 wounds ± SEM. (**E**) Mean of speed of closure of human ex vivo wounds after two days in culture ± SD ($n = 5$–7 wounds per donor). Wounds were treated twice (24 hours interval) with blue (2 J/cm^2) and red light (30 J/cm^2). $^{*}P < 0.05$, $^{**}P < 0.01$ in a two-way ANOVA.

are tempted to suggest that one cannot simply state that the expression of Opsins as a function of depth in the skin is solely guided by the wavelength-dependence of their absorption peak, as violet light absorbing OPN1-SW was also found in papillary dermal fibroblasts. As for OPN5, a UV-absorbing receptor [55], we believe that this is the first time its expression in human skin *in situ* is reported (Fig. 1). Localization of OPN5 was restricted exclusively to the basal layer of the epidermis as illustrated by its perfect co-localization with KRT14, a specific marker of basal keratinocytes. Although its peak is within the UV-A spectrum [55], which again is thought not to not penetrate deep in the skin, OPN5 was expressed in the epidermal layer containing proliferative keratinocytes, stem cells and melanocytes [56–58], where protection against UV light is critical. Since OPN5 is expressed in cultured neonatal foreskin melanocytes [41], our finding could indicate a potential role of OPN5 in the UV response of basal keratinocytes, and cross-talk with melanocytes to regulate skin protection and prevent DNA damage [59].

One could speculate that OPNs might be predominately expressed in light-exposed areas of human skin, for example, the face. This hypothesis is in agreement with the expression level of *OPN1-LW* and *OPN4* in human skin, which is higher in sun-exposed skin. However, it is not the case for *OPN5*, which was more highly expressed in non sun-exposed skin, while *OPN1-SW*, *–MW*, *OPN2* and *OPN3* did not show any difference with anatomical regions [14]. In this study, OPN1-SW, OPN3, and OPN5 demonstrated a similar pattern of localization in abdominal skin and sun-exposed facial skin (Fig. 1). The expression of OPN1-SW and OPN3 suggest they may

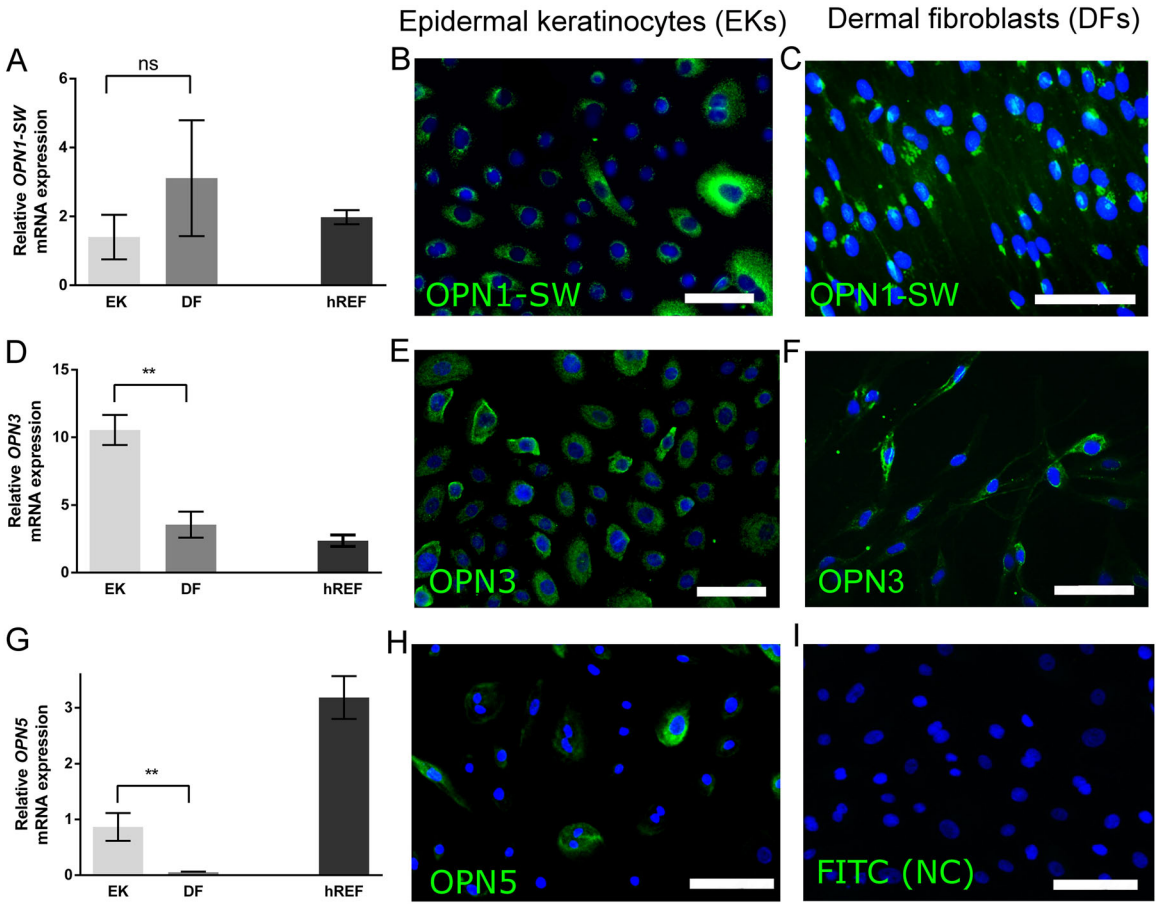

Fig. 4. Expression of Opsin mRNA and protein in cultures of human primary epidermal keratinocytes and dermal fibroblasts. Relative expression of mRNA in EKs ($n = 4$) and DFs ($n = 8$) for (**A**) OPN1-SW, (**D**) OPN3, (**G**) OPN5 ± SEM quantitated by qRT- PCR; human reference RNA (hREF) was used as a positive control. $^{**}P$-value <0.01 and ns represent not significant using Mann–Whitney test. Representative images of (**B**) OPN1-SW (green), (**E**) OPN3 (green), and (**H**) OPN5 (green) protein localization in EKs by immunofluorescence; nuclei are counterstained with DAPI (blue). Representative images of (**C**) OPN1-SW (green) and (**F**) OPN3 (green), protein localization in DFs by immunofluorescence. (**I**) Negative control (no primary antibody) in DFs. Nuclei are counterstained with DAPI (blue). Scale bar = 75 μM.

have biological functions beyond light reception in non-photoreceptive cells, or they are vestigial evolutionary remnants from mechanisms developed in the prehistoric times of "naked apes" and have remained well conserved even after humans had experienced dramatic changes in lifestyle, such as sun protection with clothing. The fact that OPN3 has been found in a range of non-photoreceptive tissues including the brain, testis and liver [10] also supports these theories. Finally, more research into the role of the expression of OPN5 in sun-exposed skin is required. The fact that OPN5 is specifically expressed only in the basal layer of the epidermis means that any sun-damage to the cells in this layer could affect the expression of OPN5.

Ex vivo cultures recapitulate the physiology of keratinocytes *in vivo as* communication with other skin cell, for example, fibroblasts, melanocytes, immune cells. An *ex vivo* human skin wound healing model was established to investigate OPNs expression in the regenerating epithelial tongue and if red or blue light could modulate wound closure. The expression of KRT17 confirmed the regeneration of a new epithelial tongue in *ex vivo* wounds (Fig. 2). OPN1-SW, OPN3, and OPN5 were all expressed in this newly formed epidermis, suggesting their role in re-epithelialization. Interestingly, OPN expression here closely resembled that of normal epidermis. While OPN1-SW was highly expressed in the apical migrating keratinocytes, OPN3 and OPN5 were highly expressed in basal keratinocytes (Fig. 3). Furthermore, expression of *OPN3* in the epithelial tongue was upregulated following exposure to low-level blue light, but not red light (Fig. 3). Since OPN3's peak absorption is in the blue spectra [15], it does not feel utterly non-logical that red light does not stimulate it. A major role of the epidermis is to provide a protective barrier against external pathogens and prevent trans-epidermal water loss (TEWL) [60]. Disruption of the

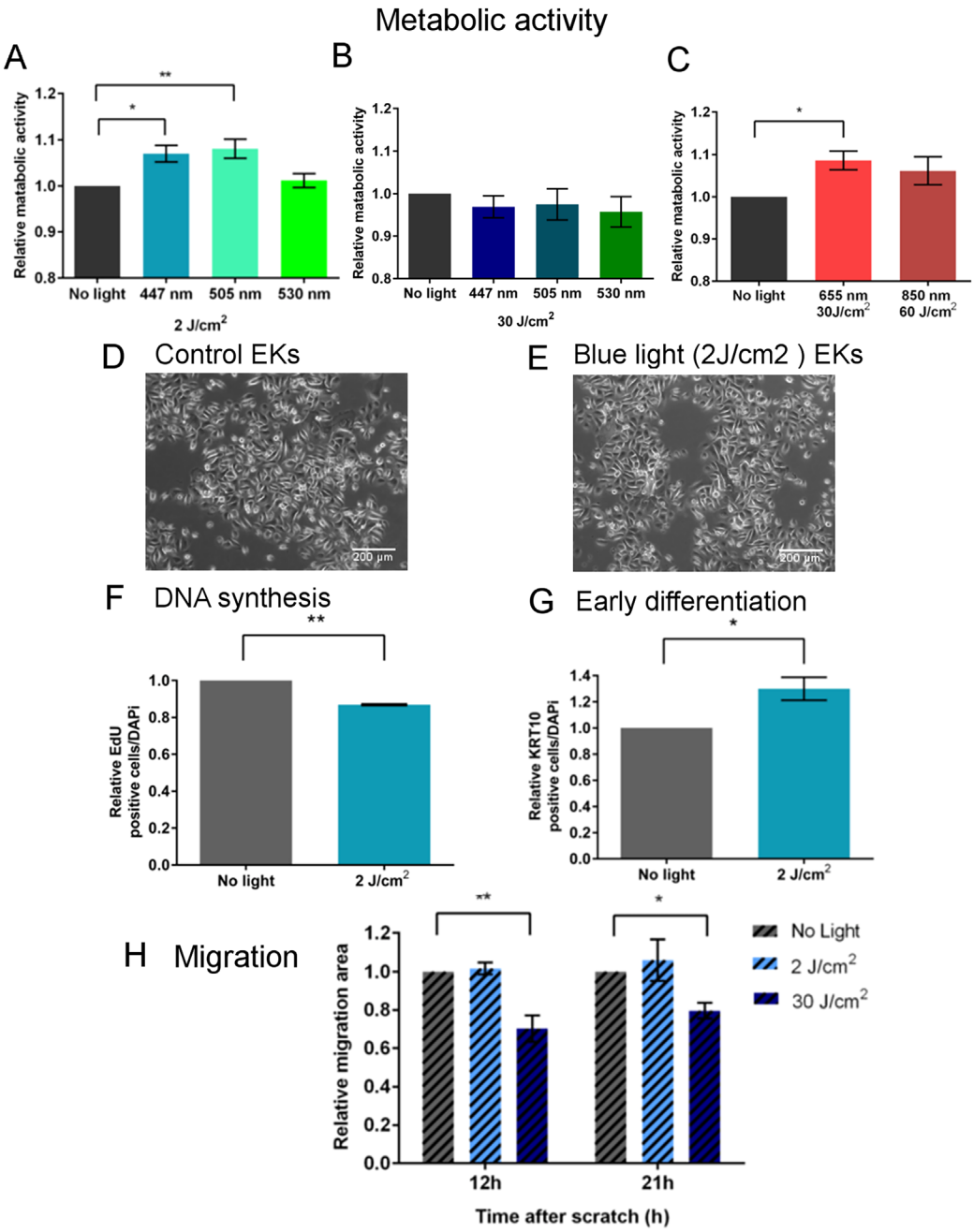

Fig. 5. Effect of visible light of different wavelengths and doses on metabolic activity, proliferation, differentiation and migration of epidermal keratinocytes. (**A–C**) Relative metabolic activity of EKs following exposure to (**A**) 2 J/cm^2 of short wavelength light 447 (blue), 505 (cyan) and 530 (green) nm, (**B**) 30 J/cm^2 of short wavelength light 447 (blue), 505 (cyan) and 530 (green) nm, and (**C**) long wavelength light (655 nm (red at 30 J/cm^2) and 850 nm (NIR at 60 J/cm^2). Data presented as mean of $n = 4$ donors ± SEM $^*P < 0.05$ and $^{**}P > 0.01$ using one way ANOVA. (**D–E**) Images of control (non-treated) (**D**) and blue light treated (2 J/cm^2) (**E**) EKs. Scale bar = 200 µm. (**F**) Quantification of DNA synthesis by EdU incorporation of EKs after irradiation with 453 nm (blue) light at 2 J/cm^2. Data presented as relative mean of EdU positive cells/DAPI ± SEM. $^{**}P > 0.01$ using unpaired t-test. (**G**) Quantification of differentiation by KRT10 expression after irradiation with 453 nm (blue) light at 2 J/cm^2. Data presented as relative mean of KRT10 positive cells/DAPI ($n = 3$ donors) ± SEM. $^*P > 0.05$ using unpaired t-test. (**H**) Quantification of migration 12 and 21 hours after scratch-wounding monolayers of EKs. EKs were irradiated with 453 nm (blue) light at 2 J/cm^2 or 30 J/cm^2 immediately after scratching. Data presented as relative mean of closure of $n = 3$ donors ± SEM. $^*P < 0.05$, $^{**}P < 0.01$ using two-way ANOVA.

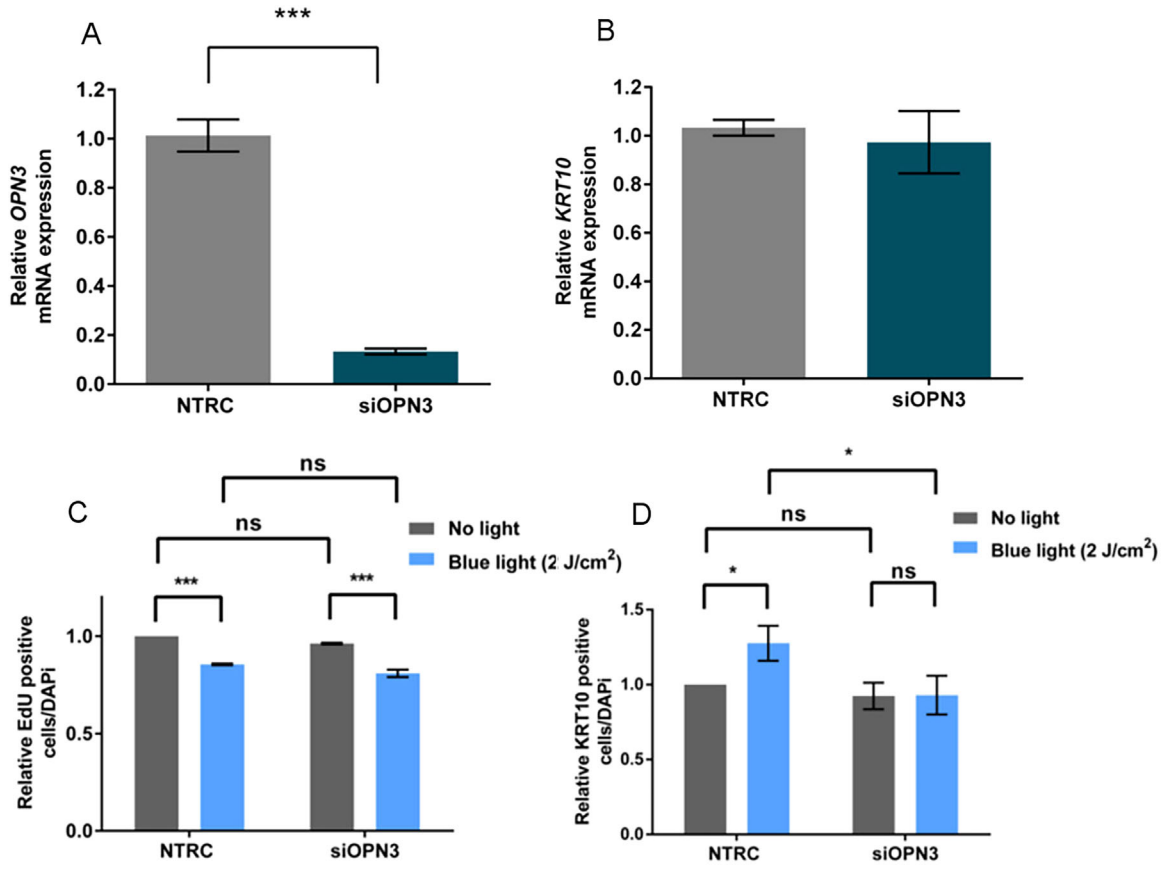

Fig. 6. Effect of blue light on proliferation and differentiation of OPN3 silenced human epidermal keratinocytes. (**A–B**) Relative mRNA expression of OPN3 (**A**) and KRT10 (**B**) in EKs treated with non-target siRNA control (NTRC) or siRNA against OPN3 for 24 hours. Data presented as mean relative expression of $n = 3$ donors ± SEM. $^{***}P > 0.001$ using a unpaired *t*-test. (**C**) DNA synthesis of EKs treated with NTRC or siRNA against OPN3 for 24 hours and irradiated with 453 nm light at 2 J/cm^2. Data presented as the mean relative EdU positive cells/DAPI of EKs ± SEM. $^{***}P < 0.001$ and ns indicates not significant using two-way ANOVA. (**D**) KRT10 expression of epidermal keratinocytes treated with non-target siRNA control or siRNA against OPN3 for 24 hours and irradiated with 453 nm light at 2 J/cm^2. Data presented as the mean relative KRT10 positive cells/DAPI of EKs ($n = 3$ donors) ± SEM. $^{*}P > 0.05$ using two-way ANOVA.

barrier leads to a series of cellular responses, including changes in gene expression. In order for keratinocytes to migrate they must undergo an epithelial to mesenchyme transition (EMT), where proteins such as vimentin are up-regulated and others, for example, E-cadherin are down-regulated [61]. While migration, proliferation and differentiation of keratinocytes is vital in wound repair, so too is restoration of the functional barrier requiring the synthesis of key components such as cross-linked lipids and proteins, for example, filaggrin and loricrin [62]. The epidermis also acts as a first-response immune organ when the barrier is breached and the upregulation of antimicrobial peptides (AMP) such as cathelicidin and defensins have also been shown to accelerate wound repair [63]. At present, a role for OPN3 in any these responses is unknown, but the increase in *OPN3* mRNA expression might indicate a functional response of OPN3 to blue light during human wound healing and warrants further investigation.

While investigating the effect of light on wound closure we noted that the differences in speed of closure highlighted donor variability in human skin *ex vivo*.

While all biopsies were taken from the same anatomical region of women of a similar age, biological variability, including hormonal fluctuations between different donors may explain the variability in wound closure [64]. Donor variability has been previously reported in skin *ex vivo* cultures, which may be attributed to different inflammatory responses induced by tissue removal [65] or different circadian rhythms as a consequence of the different environmental cues that individual donors have been exposed to (e.g. food intake, shift work, stress, smoking etc.), which can impact on epidermal stem cell function [42,66]. On a positive side, the low variation within donors (<0.017 mm^2/h, SD) supported reproducibility of the developed model. Therefore, we have chosen to study the effect of light independently in each of the donors. The response to light of different donors followed a similar

trend (Fig. 3); interestingly, low-dose blue or red light induced wound closure in Donor 2 and blue light also induced wound closure in Donor 3. Despite this observation, our thinking is that the molecular mechanisms activated by blue and red light are likely to be highly different, with CCO as the main photoreceptors of red light [2], while OPNs might be a new molecular target triggered by shorter wavelengths of light, in particular OPN3 which is suggested to be a potential light-therapeutic target as it is upregulated after blue light treatment in the epithelial tongue of the *ex vivo* wounds (Fig. 3).

In order to further investigate the molecular mechanisms of OPNs and light reception in skin cells, their expression in primary cultures of keratinocytes and fibroblasts was characterized. While mRNA expression of *OPN1SW*, *OPN3*, and *OPN5* in keratinocytes has previously been reported [41], expression in dermal fibroblasts, and expression at the protein level has not yet been established until now. We confirmed that primary keratinocytes express OPN1-SW, OPN3, and OPN5 at both the mRNA and protein level.

Furthermore, here we report for the first time that cultured dermal fibroblasts from different anatomical regions express OPN1-SW, OPN2 and OPN3, but not OPN4 and OPN5 (Fig. 4). Interestingly, expression of OPN3 mRNA was higher in keratinocytes compared to fibroblasts (which might reflect their sensitivity to light coupled to a deeper anatomical position of the latter ones and thus lower "chance" to interact with blue-colored photons), however, there was no difference in expression of OPN1-SW between these two cell types. In cultured keratinocytes and fibroblasts, OPN1-SW was confined to the peri-nuclear region. Intriguingly, the same localization profile is found in human sperm [48]. The authors of this study highlighted a role for OPN4 in sperm thermotaxis mediated by phospholipase C (PLC), due to its specific localization around the postnuclear cap, which is the location of the non-acrosomal Ca^{2+} store involved in PLC signaling. Therefore, the role of OPN1-SW in human skin may be related to temperature sensing, however, perinuclear OPN4 localization has also been observed in normal and melanoma cell lines [67], where it can respond to UV-A light in mice [14] indicating a possible photoreceptor activity of OPNs located in the perinuclear region of cells. In contrast, OPN3 was localized to the plasma membrane and cytoplasm, and OPN5 was seen in the plasma membrane and cytoplasm, however, only in keratinocytes, suggesting functional roles for non-visual OPN3 and OPN5 in human epidermis. In a first screening of OPN expression, OPN4 mRNA was not detected in primary human dermal fibroblasts in culture and therefore further study of this protein was not carried out. However, further investigation into the presence of OPN4 is still required to have the complete picture of OPN expression in human skin.

Since we identified that cultured primary cells maintained OPN expression at mRNA and protein levels, logically *in vitro* cell cultures should provide a physiologically relevant model to explore the effect of light on human skin. To determine a role in PBM, we sought to establish the effect of blue light on epidermal keratinocytes, since this is where most OPNs have their absorption spectra. What also supported our choice is that *OPN3* and *OPN5* were more highly expressed in epidermal keratinocytes than dermal fibroblasts (Fig. 3). For the sake of completeness (as highly used in PBM) red and NIR light was also included in the study on metabolic activity. Interestingly, wavelengths of blue, cyan and the red part of the spectrum (447, 505, and 654 nm) stimulated metabolic activity demonstrating the overall ability of keratinocytes to respond to visible light. Since an increase in metabolic activity was stimulated at 447 and 505 nm, but not 530 nm, this may be mediated by OPN1-SW or OPN3, as their peaks of absorption are 430 and 465 nm, respectively. One cannot exclude that OPN5 may also be involved, since its photoproduct absorbs light at 447 nm following photoconversion after UV stimulation [17].

Low-level blue light decreased DNA synthesis, but stimulated keratinocyte differentiation (Fig. 5), suggesting the increase in metabolic activity does not translate into increased proliferation, but rather into restoration of the epidermal barrier. Indeed, low-dose blue light did not modulate migration of keratinocytes, while a high dose of $30\,J/cm^2$ had an inhibitory effect, in line with reports on cultured dermal fibroblasts [24,68].

To establish whether effects of blue light on keratinocytes are modulated *via* OPNs, the consequence of silencing OPN3 was evaluated. OPN3 was not essential for DNA synthesis or differentiation of non-irradiated keratinocytes (Fig. 6). Furthermore, the decrease in DNA synthesis induced by blue light also occurred when OPN3 was silenced, suggesting the decrease in proliferation in response to blue light is not regulated by OPN3. However, blue light increased the expression of KRT10, which was abrogated by silencing OPN3, suggesting this effect is at least partially modulated by OPN3. One possible downstream mechanism triggered by OPN3 could act *via* cAMP and Ca^{2+} signaling [69], which can activate the CREB and the Ras signaling pathways. What supports this hypothesis is that knockdown of OPN3 blocked the calcium flux and the phosphorylation of CREB and Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII) in non-irradiated and blue light-irradiated human melanocytes [35]. Furthermore, OPN2 has been shown to contribute to the induction of UV-dependent Ca^{2+} mobilization in melanocytes [13]. In human epidermal keratinocytes, a non-canonical OPN2 signaling pathway mediated by Gi protein has been reported [12].

In conclusion, OPNs are expressed in human skin and the regenerating epithelial tongue after wounding, with upregulation of *OPN3* expression after blue light irradiation. Furthermore, low-level blue and red light stimulated wound closure in human skin *ex vivo*, indicating beneficial roles of light during wound healing. The effect of low-level blue light on primary keratinocytes *in vitro* highlights its potential role in regulating metabolic activity and differentiation, which is certainly of importance for epidermal barrier restoration following the re-epithelialization in wound healing. Gene silencing has identified OPN3 as a

blue-light receptor regulating keratinocyte differentiation and a potential player in restoring skin barrier functionality. Therefore blue light (at least at low dose levels) may provide beneficial effects in treatments of chronic wounds unable to restore functionality of the epidermal barrier, as well as in other skin conditions such as ichthyosis and atopic dermatitis [70].

ACKNOWLEDGMENTS

This study was supported by the European Commission 7th Framework Programme for Research and Technical Development - Marie Curie Innovative Training Networks (ITN), Grant agreement no.: 607886. The authors also acknowledge the support of the American Society for Laser Surgery & Medicine in the form of an award Best of photobiomodulation sessioñ during the ASLMS conference 2017.

AUTHORS' CONTRIBUTIONS

Dr. Irene Castellano-Pellicena designed and conducted experiments, analyzed the data and wrote the manuscript. Dr. Charles Mignon created the light-based devices and contributed to the experimental design and data interpretation of the light-based experiments. Bianca Raafs assisted in performing qRT-PCR, cell isolation and culture. Dr. ir. Natallia E. Uzunbajakava and Dr. M. Julie Thornton jointly conceived and supervised the study, assisted in data interpretation and edited the manuscript. Prof. Vladimir A. Botchkarev, MD, contributed to editing the manuscript.

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