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The activation of directional stem cell motility by green light-emitting diode irradiation

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ABSTRACT

Light-emitting diode (LED) irradiation is potentially a photostimulator to manipulate cell behavior by opsin-triggered phototransduction and thermal energy supply in living cells. Directional stem cell motility is critical for the efficiency and specificity of stem cells in tissue repair. We explored that green LED (530 nm) irradiation directed the human orbital fat stem cells (OFSCs) to migrate away from the LED light source through activation of extracellular signal-regulated kinases (ERK)/MAP kinase/p38 signaling pathway. ERK inhibitor selectively abrogated light-driven OFSC migration. Phosphorylation of these kinases as well as green LED irradiation-induced cell migration was facilitated by increasing adenosine triphosphate (ATP) production in OFSCs after green LED exposure, and which was thermal stress-independent mechanism. OFSCs, which are multi-potent mesenchymal stem cells isolated from human orbital fat tissue, constitutionally express three opsins, i.e. retinal pigment epithelium-derived rhodopsin homolog (RRH), encephalopsin (OPN3) and short-wave-sensitive opsin 1 (OPN1SW). However, only two non-visual opsins, i.e. RRH and OPN3, served as photoreceptors response to green LED irradiation-induced OFSC migration. In conclusion, stem cells are sensitive to green LED irradiation-induced directional cell migration through activation of ERK signaling pathway via a wavelength-dependent phototransduction.

1. Introduction

Light provides both illumination and thermal energy. Light exposure is physiologically responsible for human homeostasis such as vitamin D synthesis [1], sleep–wake cycle [2,3], and vision [4]. In addition, phototherapy is a standard treatment for skin disorders [5,6], neovascular retinopathy [7], or musculoskeletal disorder [8], with the uses of appropriate wavelength, intensity and duration of light exposure.

Light-emitting diode (LED) is a semiconductor light source widely used in lighting due to the advantage of high efficiency, high switching rate, and long lifetime [9]. Adjuvant phototherapy, using red or near infra-red (NIR) LED as a light source, has been demonstrated to confer therapeutic benefits on epidermis/dermis wound healing and arthritis via inhibition of inflammation [10–12]. In contrast to incandescent light sources, the multiple colors with broad spectrum wavelengths make LED light a good photostimulator to manipulate cell behavior by activating wavelength-specific photosensitizer or providing thermal energy.

Stem cells, with their self-renewal ability, multi-potency, and paracrine effect, possess a great therapeutic potential for tissue regeneration during acute injury [13]. Homing of stem cells and the

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subsequent inhibition of inflammation are critical for acute wound repair [14–16], and directional stem cell migration leads to an effective and specific tissue repair [17]. Ras homolog gene family, member A (RhoA) is essential for cell migration to regulate actin cytoskeleton reorganization and activation of extracellular signal-regulated kinase (ERK), p38 in stem cells has been reported to crucial for their migration induced by pro-inflammatory cytokines, chemokines or stromal cell-derived factor-1 [16,18–20], all of which lead to a high specificity and therapeutic efficiency of stem cells in wound repair. Orbital fat stem cells (OFSCs) are multipotent mesenchymal stem cells (MSCs) isolated from human orbital fat tissues [21], The anti-inflammation ability during acute tissue injury and tolerance in a xenotranplanted model have been demonstrated in our previous study [22]. Therefore, development of a non-invasive, light-driving method to enhance stem cell migration ability will be valuable for tissue regeneration in response to acute injury.

Up to now, only a few studies have investigated the impact of LED irradiation on stem cell behavior. Recently, red/NIR LED irradiation has been found to promote MSCs growth and enhance their osteogenic differentiation ability [23–25]. It has been demonstrated that over-expression of channelrhodopsin-2 (ChR2) in embryonic stem cells successfully differentiates into functional excitatory neuron under blue LED irradiation [26,27]. However, the mechanism(s) of red/NIR or blue LED irradiation regulating stem cell behavior is remain elucidative. Moreover, the impact of green LED irradiation on stem cell biology and the photosensitizers of green LED are still unclear.

Here we explored the effect as well as mechanism of green LED irradiation on directional migration of OFSCs. Through the comparison of migrated cells with non-migrated cells in a transwell system under green, red LED light exposure or heat, the mechanism controlling green LED irradiation-induced cell migration and the role of adenosine triphosphate (ATP) in OFSC migration were elucidated. The photosensitizers responsible for green LED irradiation-induced motility in OFSCs were also delineated in this study.

2. Materials and methods

2.1. Cells Isolation and culture

OFSCs were isolated from human orbital fat tissues with informed consent. Approval from the Institutional Review Board of Wan Fang Hospital, Taipei Medical University was obtained prior to the commencement of the experiments. The procedures of isolating OFSCs were described previously [21]. Briefly, adipose tissues were fragmented, digested, and filtered. After centrifugation, the cells from the resulting pellet were plated in non-coated tissue culture flasks (BD Biosciences, San Jose, CA, USA) with MesenPro medium (Invitrogen, Carlsbad, CA, USA). Cells with colony formation ability, MSC surface phenotype profile, and tri-linage differentiation capacity were defined as OFSCs. The OFSCs were maintained in MesenPro medium (Invitrogen) under non-contact culture as described previously [28]. Briefly, cells from one flask were detached when their density reached 60–70% of confluence, and were re-seeded into three new flasks. Eighth to tenth passage of OFSCs were used for the experiments.

SV-40 immortalized human corneal epithelial (HCE-T) cells [29] were kindly given by Dr. Araki-Sasaki. The cells were cultured in DMEM/HamF12 (1:1) medium supplemented with 5% fetal bovine serum (HyClone, Logan, UT, USA), 5 μg/ml insulin, 0.1 μg/ml cholera toxin (Sigma–Aldrich, St. Louis, MO, USA), 10 ng/ml recombinant human epidermal growth factor (hEGF) (BD Biosciences), and 0.5% DMSO, as previous description [30].

2.2. LED photosystem

The LED photosystem is illustrated in Fig. 1. OFSCs (7500 cells/transwell) or HCE-T cells (5000 cells/transwell) were seeded on the upper surface of 8 μm pore sized transwell culture plate (PI8P01250, Millipore Millicell® cell culture insert, Millipore, Billerica, MA, USA). The attached cells were covered by undifferentiated medium (MesenPro, Ivitrogen) (Fig. 1B) and exposed to a 530-nm green LED (M530L2, Thorlabs, Inc., Newton, NJ, USA) or a 625-nm red LED (M625L2, Thorlabs) light at a distance of 30 cm away from LED light source (Fig. 1A). The full power density of LED irradiated on cells was 66.4 $\mu W/cm^2$ and the power density could be reduced to 22.8 (filter 1) or 11.3 (filter 2) $\mu W/cm^2$ by a polariscope at 6 cm from the light. Cells in the transwell system under dark at the same time points served as the time-matched control.

2.3. Cell migration assay

The cells in the transwell system were under LED irradiation or dark control with or without 25 μM of ERK inhibitor (328005, Merck, Whitehouse Station, NJ, USA) for 24 or 48 h. In this transwell migration system, migrated cells were defined by cells migrating to the bottom surface of transwell plate, while non-migrated cells were defined by cells remaining on the upper surface of transwell plate.

For migrated cell (or non-migrated cell) counting and staining, cells on the upper surface (or on the bottom surface) of transwell culture plate were totally removed by a scraper. Cells migrated to the bottom surface (or remained on the upper surface) were fixed by 3.7% formaldehyde for 20 min, and then stained by TRITC-labeled phalloidin (1:500; Sigma–Aldrich, St. Louis, MO, USA) at 37°C for 1 h, followed by 4-,6-diamidino-2-phenylindole (DAPI) for 5 min. Migrated cells on the bottom surface were counted under a fluorescence microscopy (Leitz, Germany) with 100× magnification. The migrated cell number in each sample was determined, and the mean value was determined from cell numbers in ten random fields. More than three independent experiments were performed for each condition.

2.4. Cell proliferation assay

Cells were seeded in a 96-well plate (2000 cells/well) for 4 h before being exposed to LED light or ERK inhibitor. After green LED irradiation for 24, 48, 72 h or incubation with ERK inhibitor (328005, Merck) for 48 h, the culture medium in each well was then replaced by 100 μl of serum-free DMEM and 20 μl MTS reagent (Promega, Madison, WI, USA). The signal at OD490 was measured by using a microplate reader (Bio-Rad, Hercules, CA, USA) after cells were incubated in the dark at 37 °C for another 1–4 h.

2.5. Gene expressions

RNAs were extracted using the RNeasy Kit (Qiagan Inc., Valencia, CA, USA) followed by reversely transcribed to cDNA using an Advantage RT-for-PCR kit (Clontech, Palo Alto, CA, USA). cDNA was amplified using a Mastercycler Gradient 5331 Thermal Cycler (Eppendorf, Germany). For microarray analysis, the differential gene expressions were detected by GeneChip™ (Affymetrix, Santa Clara, CA, USA) and analyzed by Affymetrix Microarray Suite 5.0. For real-time RT-PCR, gene expression level was represented by monitoring the fluorescence signals after each cycle with an ABI 7300 Real-Time PCR system (Applied Biosystems). Primers used for real-time RT-PCR were listed in Table 1.

2.6. Intracellular ATP content

Intracellular ATP level was measured using ATP determination kit (Invitrogen) as per the manufacturer's instructions. Briefly, cells were trypsinized, resuspended in CelLytic™ MT mammalian tissue lysis/extraction reagent (100 ul; Sigma–Aldrich) to release the intracellular ATP. The supernatant (10 μl) was then transferred into a 96-well cell culture cluster (Corning Costar, NY, USA) containing 90 μl ATP standard reaction solution and measured by Luminoskan Ascent Luminometer (Thermo Electron Corp., Waltham, MA, USA).

2.7. Intracellular kinase activity

Intracellular kinase activity was determined by using human phospho-kinase array kits (R&D system, Minneapolis, MN, USA) and Western blot analysis according to the manufacturer's instructions. Briefly, cells were trypsinized and resuspended in lysis buffer to obtain the cell lysate. For kinase array, each cell lysate (300 μg) was incubated individually with the antibody-pre-coated membrane overnight. After washing, the membrane was incubated with biotinylated-labeled antibody cocktail for 2 h, and then incubated with streptavidin-HRP for another 30 min.

For Western blot analysis, 30 μg of protein was separated on 10% SDS-PAGE and blotted onto PVDF membrane (Amersham Biosciences, Uppsala, Sweden), followed by blocking with 5% skim milk in TBST buffer (50 mm Tris–HCl, 150 mm NaCl, 0.1% Tween 20, pH 7.4). The membrane was then blotted with indicated primary antibodies such as ERK (1:1000, Cell signaling, Danvers, MA, USA), p-ERK (1:2000, Cell signaling) and α-tubulin (1:10000, Sigma–Aldrich). After 3 times of washes, the membrane was incubated with the HRP-conjugated secondary antibody (1:5000, Santa Cruz, Santa Cruz, CA, USSA). The signals on each membrane was detected by ECL chemiluminescent reagent (PerkinElmer Life Sciences, Inc.) and their intensities were quantitatively measured by a densitometry (LabWorks, UVP Inc., Upland, CA, USA).

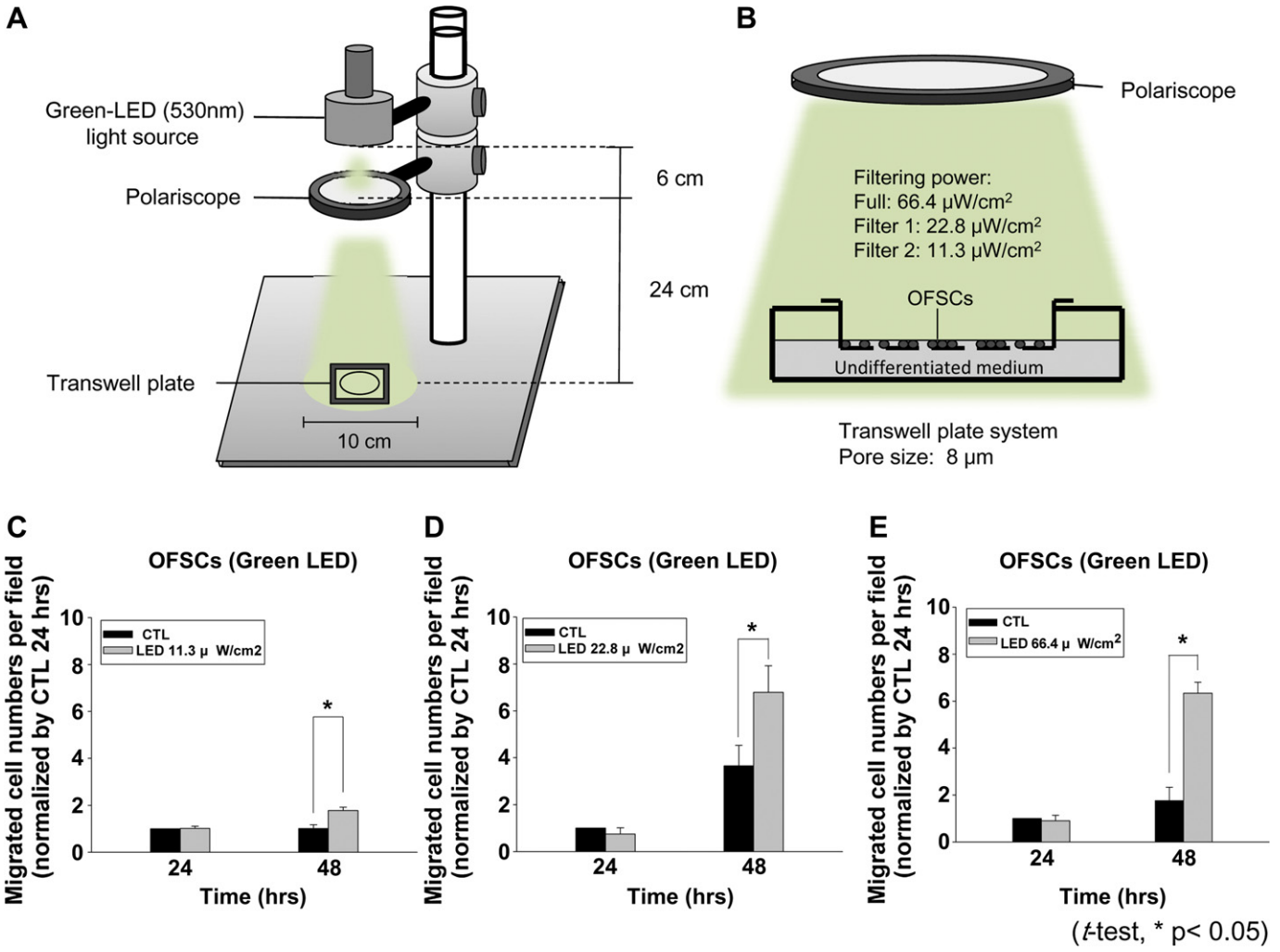

Fig. 1. Green light-emitting diode (LED) irradiation enhanced human orbital fat stem cell (OFSC) migration (A) Schema of LED photosystem. (B) Schema of cells in transwell migration system under LED irradiation. Green LED enhanced OFSC migration ability after 48-h irradiation with power density of 66.4 (C), 22.8 (D) and 11.3 (E) μW/cm^2.

2.8. Temperature measurement

Temperature of medium was measured under dark or LED light exposure using high-sensitivity glass probe thermistor (SP31A, Sensor Scientific, Inc., Fairfield, NJ, USA). Temperature was recorded every 2 h, and calibration of thermistors was performed before temperature measurement at each time point according to the manufacturer's instructions.

Table 1
Primers for real-time reverse transcription-Polymerase Chain reaction.

Gene	Primer Sequence	Product (bp)
RRH	F: 5′-ACCACCAACACTTACATCGG-3′ R: 5′-TAGCACCAGTAGGATCTGGG-3′	112
OPN1SW	F: 5′-ATGGGCCTCAGTACCACATT-3′ R: 5′-GCAACTTTTTGTAGCGCAGT-3′	132
OPN3	F: 5′-TCAGTGCACAATGGCTAGAG-3′ R: 5′-GCGGTTCCCCGAGTACAT-3′	135
OPN5	F: 5′-TTGGGAAGCGGATTTAGTGG-3′ R: 5′-TTATTTCAGCGGGTCTCAGC-3′	125
ChR2	F: 5′-CTCACGCGCTGCAATGTCCT-3′ R: 5′-GCAGCCGAGTGGGGTAATGC-3′	113
RhoA	F: 5′-CAGAAAAGTGGACCCCAGAA-3′ R: 5′-TGCCTTCTTCAGGTTTCACC-3′	147
18S rRNA	F:5′-ATGGCCGTTCTTAGTTGGTG-3′ R:5′-AACGCCACTTGTCCCTCTAA-3′	132

2.9. Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Science software (v. 16, SPSS Inc., Chicago, IL). Differences in viability with and without ERK inhibitor, kinase activities, temperature or migrated cell numbers between LED irradiation and dark control at the same time point were assessed using the two-tailed, non-paired *t*-test, and for significance, *P*-values < 0.05. Differences among the migrated cells numbers and intracellular ATP content at various temperatures, viability or migrated cell numbers with and without LED irradiation at various time points, or data from the gene expression levels (i.e. RhoA, retinal pigment epithelium-derived rhodopsin homolog (RRH), short-wave-sensitive opsin 1 (OPN1SW), and encephalopson (OPN3)), and ATP content in migrated and non-migrated cells with and without LED irradiation were assessed using analysis of variance, Tukey's post-hoc test, and a 95% confidence level. Different characters represented different levels of significance, and level "ab" indicated statistical level in between level "a" and level "b". Error bars shown in all figures represent standard deviation of mean values.

3. Results

3.1. Effect of green LED irradiation on OFSC migration

The study design is illustrated in Fig. 1A and B. The orbital fat stem cells were initially seeded on the upper surface of the transwell plate. The cells that have migrated to the bottom surface of the transwell plate are designated as migrated cells,

while non-migrated cells are cells remaining on the upper surface. Compared to time-matched dark control, 48 h of green LED irradiation significantly increased the migrated cell numbers under the power densities of 11.3 (Fig. 1C), 22.8 (Fig. 1D) and 66.4μW/cm^2 (Fig. 1E), but such increases were not seen at 24 h (Fig. 1C–E), nor at 6 or 12 h (data not shown).

Actin cytoskeleton re-organization is essential for cell migration [31] and the formation of actin stress fibers during cell migration is regulated by RhoA [32,33]. After 48 h of green LED irradiation, F-actin signals in migrated OFSCs (Fig. 2A, right) were stronger than in migrated cells under dark (Fig. 2A, left). Green LED irradiation significantly up-regulated RhoA expression in migrated OFSCs, but did not alter the RhoA expression in non-migrated cells (Fig. 2B).

3.2. Effect of green LED irradiation on OFSC proliferation

To determine whether green LED irradiation affected cell proliferation, the numbers of viable OFSCs under green LED light exposure at various time points were measured by MTS assay. As shown in Fig. 2C, OFSC number doubled between 48 and 72 h both in the dark and under green LED exposure; thus, LED irradiation showed no effect on OFSC proliferation.

To avoid the contribution of cell division to increase in migrated cell numbers, we performed the following experiments under LED exposure for up to 48 h at the power density of 66.4μW/cm^2.

3.3. Effect of green LED irradiation on HCE-T cell migration

To test the cell specificity of the induction of migration by green LED irradiation, OFSCs were replaced by HCE-T cells, which are well-differentiated corneal epithelial cells, in the LED photosystem. HCE-T cell migration was also enhanced under 66.4 μW/cm^2 of green LED irradiation for 48 h, but the increase over this time period was less than 1.5 fold (Fig. 3A), as compared with the 3-fold increase for the OFSCs (Fig. 1C). Light-driven F-actin reorganization in migrated OFSCs (Fig. 2A) was also more prominent than in migrated HCE-T cells (Fig. 3B).

3.4. Role of wavelength on LED irradiation-induced cell migration

To further dissect the role of wavelength in such a photo-induced cell migration, green LED (530 nm) was replaced by red LED (625 nm), which has no overlap of spectrum wavelength with the green LED light. Surprisingly, under the same power density to green LED irradiation (66.4 μW/cm^2), red LED irradiation for 48 h neither affected OFSC (Fig. 3C) nor HCE-T cell (Fig. 3D) migration in the photosystem.

3.5. ATP content in green LED irradiated OFSCs

Migration is an energy consumption behavior for a cell. ATP is essential for phosphorylation of most protein kinases [34] and increases the formation of guanosine triphosphate (GTP) by transferring phosphate to GDP under light stimulation [35]. Besides, ATP may form cyclic adenosine monophosphate (cAMP) by conformational change [34]. We further investigated the effect of green LED irradiation on ATP production and OFSCs migration. As shown in Fig. 4A, green LED irradiation markedly increased ATP production in both migrated and non-migrated OFSCs. Compared to non-migrated cells, however, the intracellular ATP content in migrated OFSCs was significantly lower, suggesting that ATP was utilized during cell migration.

3.6. Alteration of temperature in green LED irradiated OFSCs

Thermal stress has been reported to increase ATP production from mitochondria [36]. Monitoring the temperature in the culture medium at various time points under green LED irradiation and dark control showed that green LED irradiation significantly elevated local temperature after 8 h of light exposure. In average,

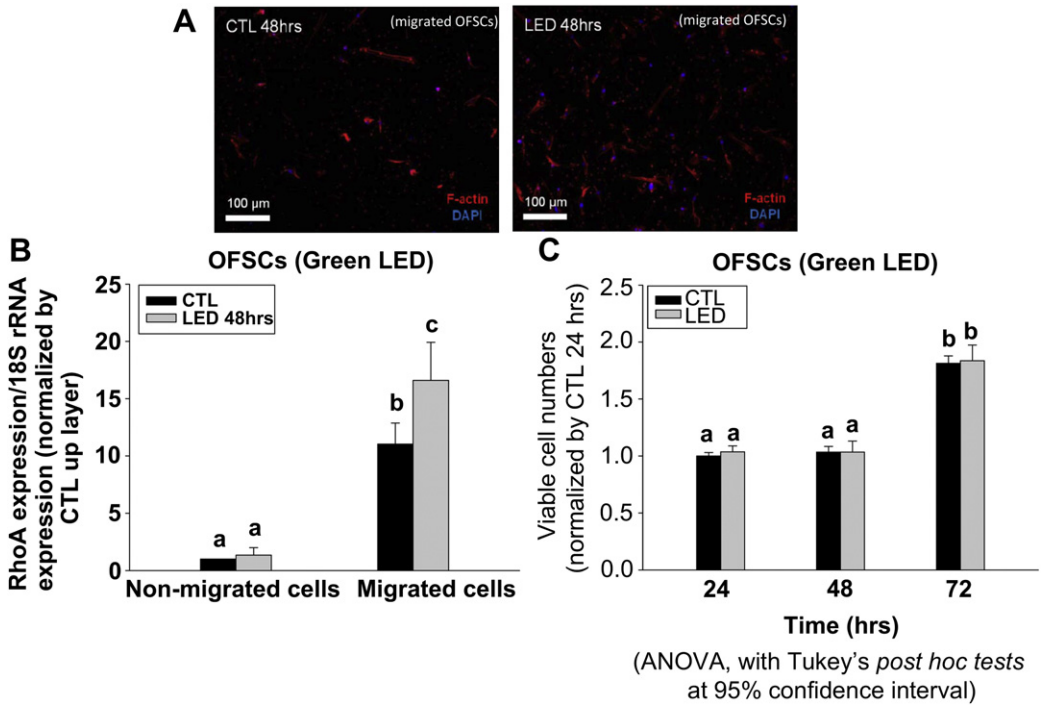

Fig. 2. Green LED irradiation-induced OFSC migration was associated with F-actin reorganization, and not a consequence of cell division (A) F-actin signal in migrated OFSCs under green LED irradiation (right) was stronger than which in migrated OFSCs under dark control (left). (B) RhoA expression was up-regulated in migrated OFSCs, and green LED irradiation further increased RhoA expression in migrated OFSCs. (C) The doubling time of OFSCs was in between of 48–72 h, and green LED irradiation did not affect OFSC viability.

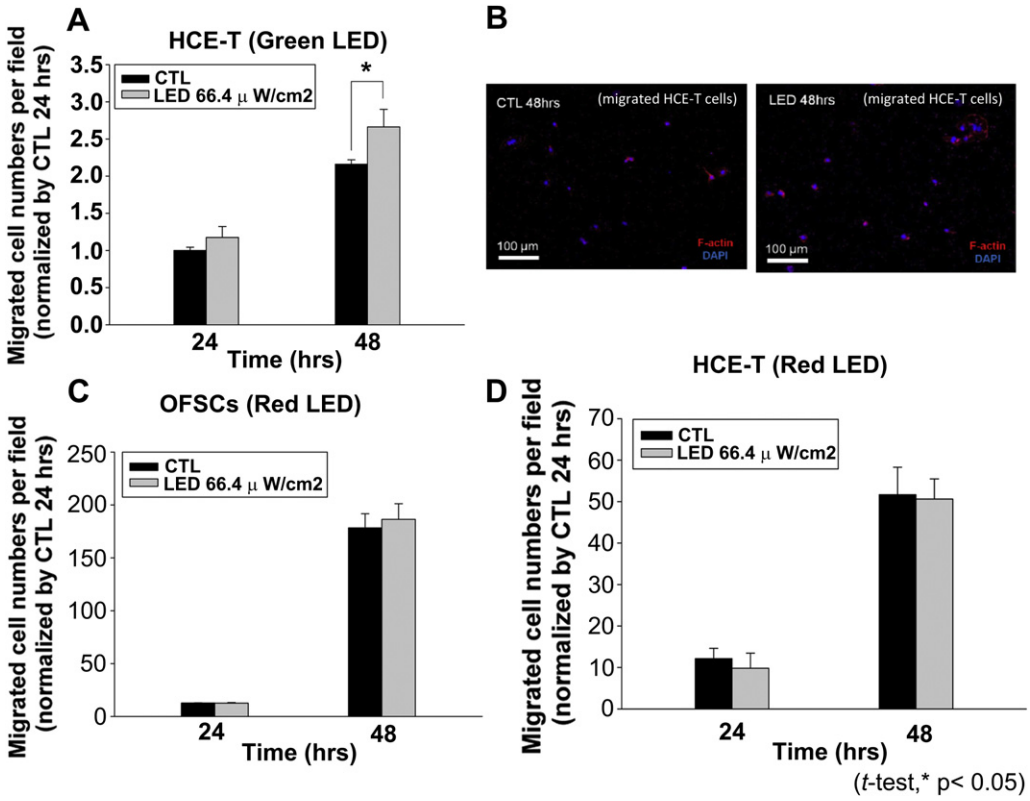

Fig. 3. LED irradiation-induced cell migration was wavelength specific green LED irradiation enhanced human corneal epithelial (HCE-T) cells migration (A), and F-actin signal in migrated HCE-T cells was not significantly increased by green LED irradiation (B). Red LED irradiation neither altered the migration ability in OFSCs (C) nor in HCE-T cells (D).

the medium temperature under green LED irradiation (37.4 °C) was only 0.1 °C higher than dark control (37.3 °C) in the first 48 h (Fig. 4B), and which was associated with increase in 100% of ATP production in OFSCs (Fig. 4A).

3.7. Thermal effect on ATP production and cell migration

To determine the role of thermal effect on ATP production and cell migration, we increased culture medium temperature by heat instead of LED light for 48 h. As showed in Fig. 4C–F, responses of ATP production and cell migration to thermal effect could be observed only in OFSCs (Fig. 4C and D), but not HCE-T cells (Fig. 4E and F). Elevation of 1.0 °C by heat was associated with a 30% increase of ATP production in OFSCs (Fig. 4C and D), and this temperature effect on ATP production was not seen in HCE-T cells (Fig. 4E and F). However, elevation of 0.1 °C (from 37.3 °C to 37.4 °C) by heat did not alter the migration potential of OFSCs (Fig. 4G), indicating that the green LED irradiation-induced ATP production and cell migration were not attributable to thermal stress.

3.8. Phototransduction for green LED irradiation-induced OFSC migration

Since green LED irradiation increased ATP production, and ATP was utilized for OFSC migration (Fig. 4A), we looked for the target(s) of increased ATP during cell migration promoted by green LED irradiation. Human phosphorylated kinase array was performed to analyze the differential kinase activities in OFSCs between green LED irradiation and dark control. We found that eight of the kinases, i.e., p38, ERK 1/2, c-Jun N-terminal kinase (JNK), MAP kinase kinase (MEK) 1/2, Akt, cAMP response element-binding (CREB) (Fig. 5A), Yes (Fig. 5B), and c-Jun (Fig. 5C) were markedly activated after 48-h green LED irradiation, while three of the kinases, i.e., Lyn, signal transducer and activator of transcription (STAT) 5 alpha (Fig. 5B), and p70S6 kinase (Fig. 5C) were inhibited. Kinases involved in cell cycle, such as p53, p27 and TOR, were not affected by green LED irradiation (Fig. 5D).

To assess the role of ERK signaling pathway in the green LED irradiation-induced OFSC migration, intracellular ERK activity was inhibited by using an ERK activation inhibitor peptide II that targets the phosphorylation of ERK (Fig. 6A). OFSC viability was not affected by the ERK inhibitor (25 μM) over the 48 h of study (Fig. 6B). The inhibition of ERK activity selectively abrogated the green LED irradiation-induced OFSC migration, but had no effect on cell migration in the dark control (Fig. 6C). The ERK inhibitor also decreased F-actin signals in the migrated cells triggered by green LED irradiation (Fig. 6F and G) and did not affect F-actin in the migrated cells under dark (Fig. 6D and E).

3.9. Photosensitizers for green LED irradiation-induced OFSC migration

To find out the putative photosensitive molecules that directed the green LED irradiation-induced OFSC migration, microarray analysis was used for screening the basal expression level of opsins (OPNs), the photosensitizers in animal cells [37], in OFSCs. As shown in Table 2 listing the human opsins have been identified, gene expressions of RRH, OPN1SW, OPN3 and OPN5 were detectable by at least one probe on the microarray chip. OFSCs did not express rhodopsin (OPN2), long/medium-wave-sensitive opsin 1 (OPN1LW/OPN1MW), ChR2, melanopsin (OPN4), neuropsin (KLK8), or retinal G protein coupled receptor (RGR). Real-time RT-PCR was

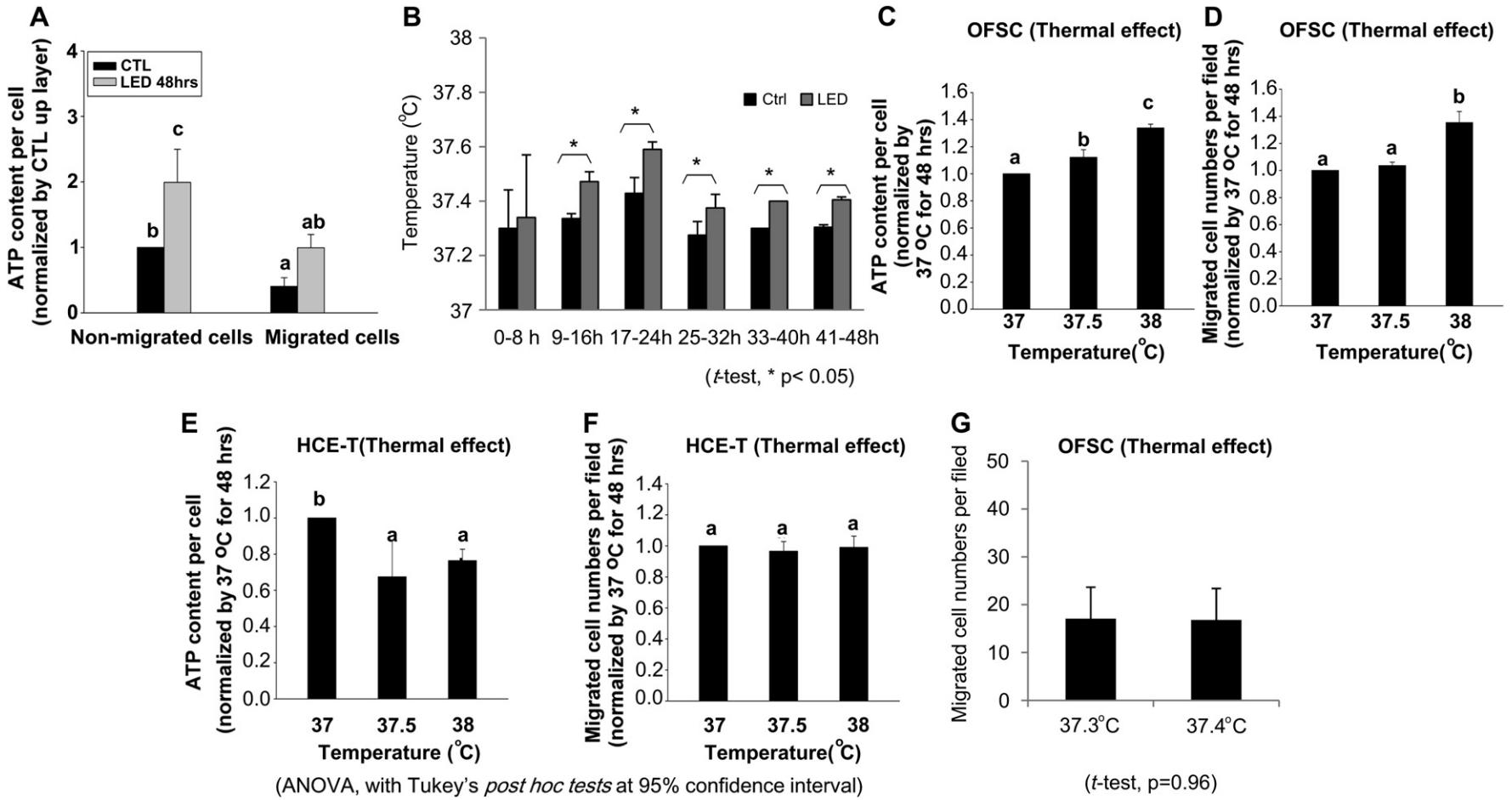

Fig. 4. Thermal effect partially contributed in green LED irradiation-induced ATP production for OFSC migration (A) green LED irradiation increased intracellular ATP production by 2 folds in both migrated and non-migrated OFSCs, and ATP consumption was observed during cell migration. (B) Green LED irradiation increased the temperature by 0.1 °C after 8 h of light exposure. (C) Elevation of temperature by heat up to 0.5 °C and above significantly increased ATP content in OFSCs. (D) Elevation of temperature by heat up to 1 °C significantly increased OFSC migration. Heat-induced thermal effect neither changed the ATP content in HCE-T cells (E) nor HCE-T motility (F). Elevation of temperature from 37.3 °C to 37.4 °C by heat did not alter the migration potential in OFSCs (G).

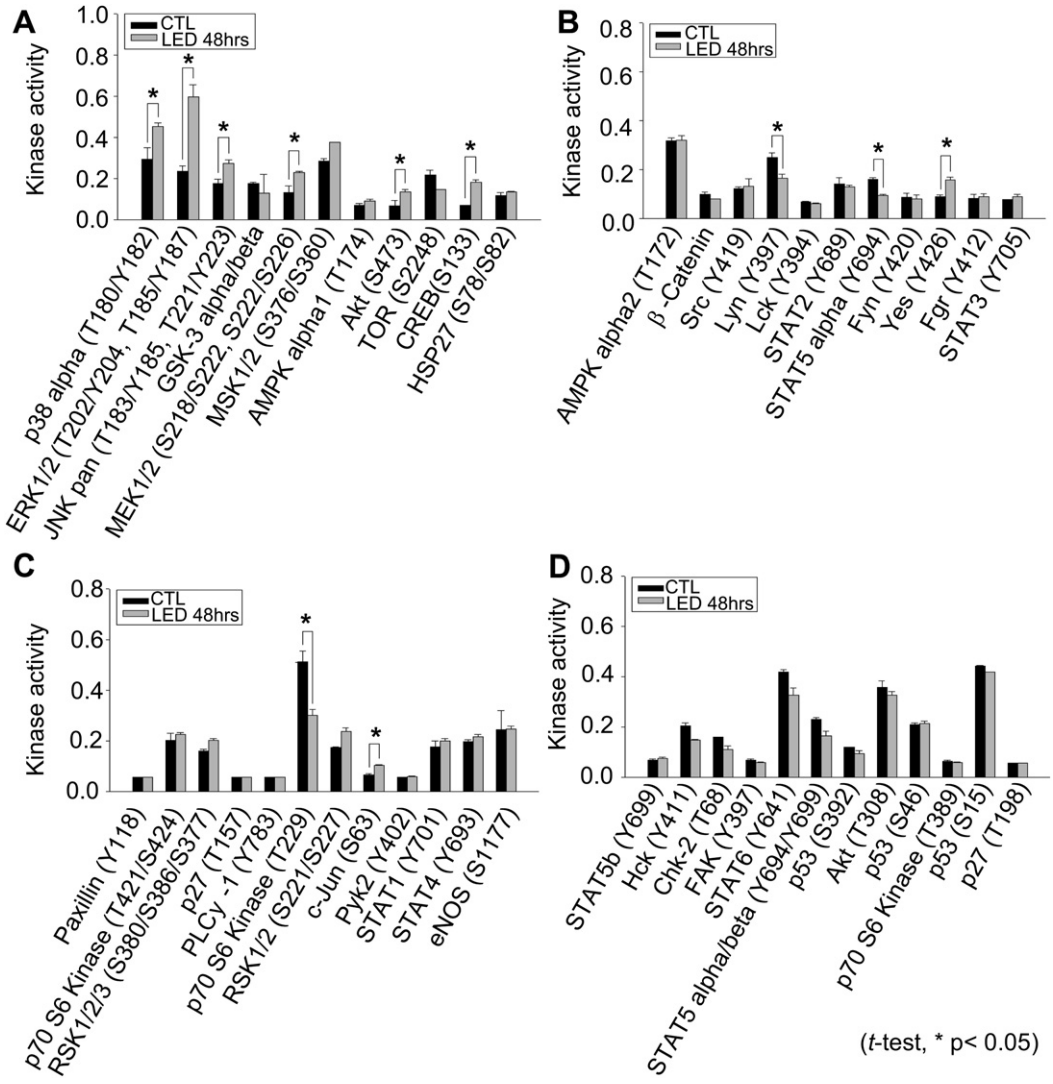

Fig. 5. Green LED irradiation significantly activated ERK/MAPK/p38 signaling pathway in OFSCs after 48 h of green LED irradiation, intracellular kinase activities of p38, ERK1/2, JNK, MEK1/2, Akt, CREB (A), Yes (B), and c-Jun (C) were significantly increased, while Lyn, STAT 5 alpha (B), and p70S6 kinase (C) were decreased. (D) Kinases involved in cell cycle such as p53, p27 and TOR were not affected.

performed on the photosensitizers detected by microarray under dark and green LED irradiation for 48 h; the results confirmed the constitutional expression of RRH, OPN1SW and OPN3, but not OPN5 (data not shown). Using transwell migration assay, we found upregulation of RRH (Fig. 7A) and OPN3 (Fig. 7B), but not OPN1SW (Fig. 7C), during the first 24 h in migrated OFSCs triggered by green LED irradiation. In the dark control, the expressions of RRH (Fig. 7A) and OPN1SW (Fig. 7C) were not significantly different between non-migrated and migrated OFSCs. OPN3 expression was decreased in migrated OFSCs under dark control in comparison of non-migrated OFSCs (Fig. 7B).

4. Discussion

In this study, we demonstrate that green LED (530 nm) irradiation triggers directional stem cell motility through activation of phototransduction mediated by the ERK signaling pathway. The enhancement of OFSC migration by green LED irradiation is wavelength specific, but not limited to OFSCs. OFSCs, stem cells isolated from orbital fat tissue, are more sensitive to light-induced migration than the differentiated HCE-T cells. Green LED irradiation increases ATP production to facilitate ERK/MAPK/p38 kinase phosphorylation in OFSCs. RRH and OPN3 are photosensitizers in OFSCs that respond to green LED irradiation, which induced cell migration away from the light source.

Directional migration of stem cells results in an effective and specific tissue repair [17]. In this study, kinases involving the ERK/MAPK/p38 signaling pathway were selectively activated by green LED irradiation (Fig. 5), and inhibition of ERK phosphorylation selectively abrogated the green LED irradiation-induced OFSC migration (Fig. 6), showing that the migration enhancement by LED irradiation is a consequence of the activation of the ERK/MAPK/p38 signaling pathway. It has been reported that light exposure may induce activation of ERK/MAPK/p38 signaling pathway in tissue cells, such as human endothelial cells [38], skin fibroblast [39] and mouse epidermal cells [40], and it is known that activation of this signaling pathway is crucial for directional stem cell migration [16,18–20]. Our data indicate that green LED irradiation enhanced directional cell motility away from the light source through this signaling pathway axis (Figs. 1, 2 and 5). Further studies to evaluate the therapeutic effect of green LED-irradiated OFSCs on tissue repair are warranted.

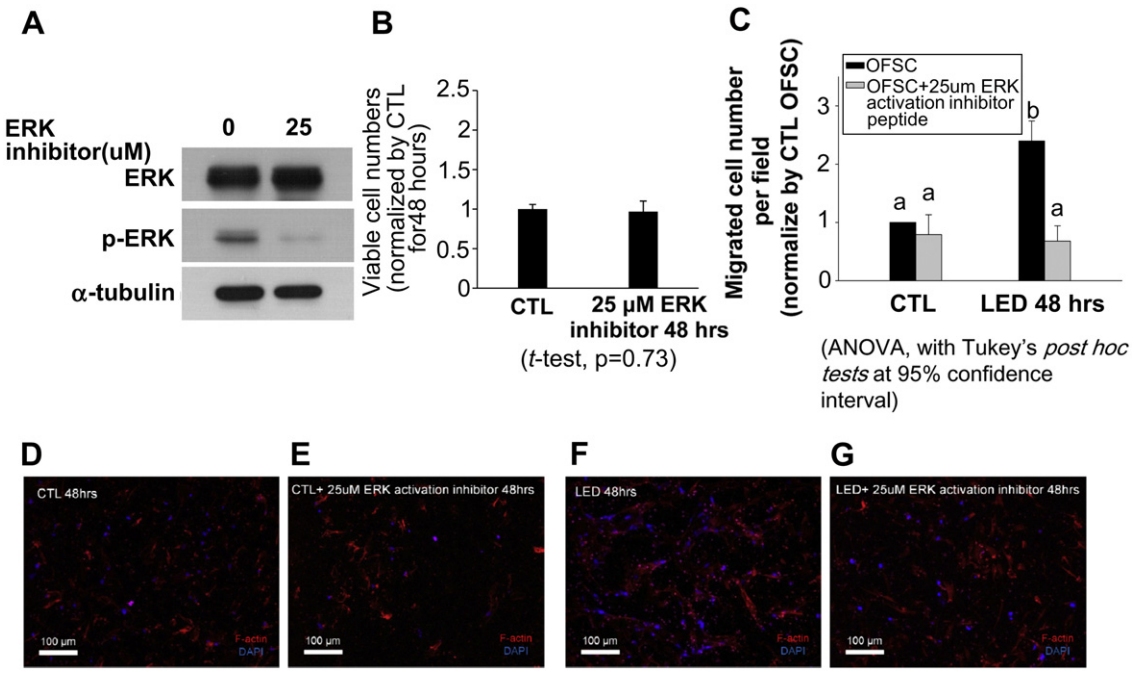

Fig. 6. Inhibition of ERK activity abrogated green LED irradiation-induced OFSC migration (A) Twenty-five μM ERK inhibitor blocked ERK phosphorylation in OFSCs within 2 h. Persistent treatment of 25 μM ERK inhibitor did not affect OFSC viability (B), but totally abrogated green LED irradiation-induced OFSC migration in the first 48 h (C). (D, E) Under dark control, inhibition of ERK activity did not change F-actin organization in migrated OFSCs. (F, G) Under green LED irradiation, inhibition of ERK activity significantly affected F-actin re-organization in migrated OFSCs.

Therapeutic effect of red/NIR LED irradiation via thermal effect-induced ATP production has been reported in the literature. Elevation of local temperature during red/NIR LED or laser phototherapy leads to increase ATP content in the treated animal brain tissue [41,42], suggesting that thermal effect is one of the mechanism(s) of red/NIR LED phototherapy. In this study, OFSCs were more sensitive to thermal effect on ATP production and cell motility than HCE-T cells (Fig. 4C–F). However, green LED irradiation only minimally increased the local temperature (Fig. 4B), and this slight increase in temperature was not sufficient to increase the ratio of ATP production (Fig. 4G), indicating that temperature does not play a role in green LED irradiation-induced ATP production and cell migration.

ATP is essential for the phosphorylation of most protein kinases [34]. Green LED irradiation increased ATP production in OFSCs (Fig. 4A) accompanied by activation of ERK/MAPK/p38 kinase activities (Fig. 5) and migration enhancement (Fig. 1), suggesting that the increase in ATP production may facilitate ERK 1/2, MEK, p38, JNK, and c-Jun phosphorylation for OFSC migration. However, the kinase phosphorylation selectivity by ATP requires appropriate photosensitizer-triggered phototransduction.

Table 2
Gene expression of photosensitizers in OFSCs.

Gene symbol	Gene Title	Expression in OFSCs
RRH	retinal pigment epithelium-derived rhodopsin homolog	+-
RHO	rhodopsin (opsin 2, rod pigment)	-
OPN1SW	short-wave-sensitive opsin 1 (cone pigments)	+-
OPN1LW/ OPN1MW	long/medium-wave-sensitive opsin 1 (cone pigments)	-
ChR2	channelrhodopsin-2	-
OPN3	opsin 3 (encephalopsin, panopsin)	++
OPN4	opsin 4 (melanopsin)	-
OPN5	opsin 5	+-
KLK8	kallikrein 8 (neuropsin/ovasin)	-
RGR	retinal G protein coupled receptor	-

++: gene expression can be detected by two independent probes.
+: gene expression can be detected by one of two independent probes.
-: negative gene expression.

Opsins are photoreceptors in animal cells and there are more than sixty opsins have been identified. Most opsins are chromophore containing trans-membranous G-protein coupled receptors, and cellular signal delivery follows specific opsin activation by light so that cell behavior may altered by photoirradiation [37]. Among human opsins, it can be divided into visual opsins and non-visual opsins. Visual opsins are photorsensitizers expressing in photoreceptor cells, i.e. OPN1LW, OPN1MW and OPN1SW to mediate wavelength-specific phototransduction for color vision in cone cells, and OPN2 in rod cells for night vision [43–45]. Non-visual opsins are phosensitizers such as OPN3, OPN4, OPN5, RRH, KLK8 and RGR. OPN3 (encephalopsin or panopsin) is strongly expressed in brain and testes [46,47], but functional unclear. Recently, OPN3 expression in lung bronchial epithelia and immune cells has been reported to associate with asthma and modulation of T-cell response [48]. OPN4 are located in retinal ganglion cells and retinal pigment epithelial cells for pupillary light response, light entrainment of the circadian rhythm, and photopigment regeneration [49–51]. RRH, OPN5 and RGR are opsins expressing in retina, and may encode a protein with photoisomerase activity [52–54].

Up to now, what kinds of opsins are expressed in stem cells, as well as their functions in stem cells, has not been reported in the literature. Recently, OPN1SW, OPN2, OPN3, OPN4, OPN5, and RRH expressions are found in spontaneously immortalized human Müller cell lines exhibiting retinal progenitor characteristics [55], but the function of those opsins in retinal progenitor cells needs be defined. It is well accepted that animal vision starts with cAMP signaling mediated by opsin-G protein cascade [56]. In hippocampus, light/dark cycle with oscillation reactivates ERK1/2, MAPK and cAMP/CREB signaling pathway is critical for persistent

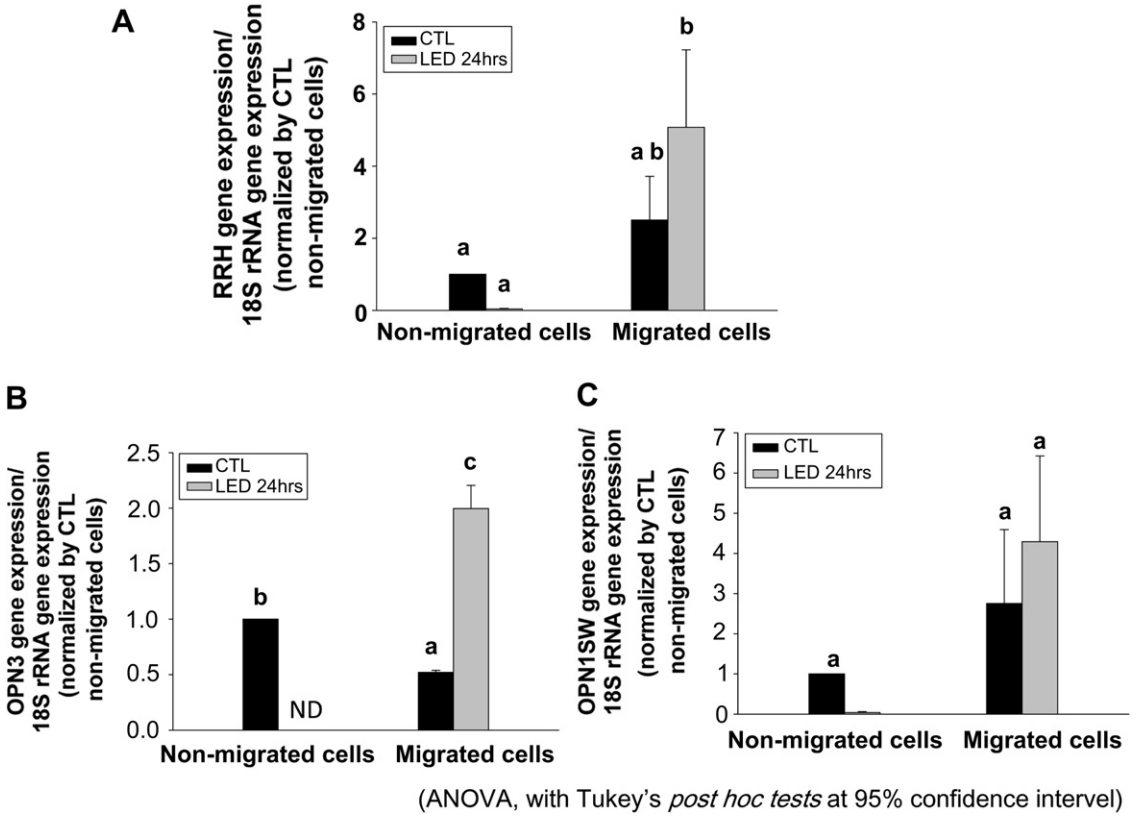

Fig. 7. Upregulation of RRH and OPN3 in OFSCs accompanied green LED irradiation induced cell motility OFSCs expressed three photosensitizers, which were RRH (A), OPN3 (B) and OPN1SW (C). After green LED irradiation, gene expressions of RRH (A) and OPN3 (B) were upregulated in migrated OFSCs, but not in non-migrated OFSCs. (C) OPN1SW was not sensitive to green LED irradiation.

of long-term memory [57]. In this study, green LED irradiation-activated CREB, ERK1/2 and MAPK (Fig. 5A) supported that activation of ERK/MAPK/p38 pathway may a consequence of light-induced opsin-G protein cascade. OFSCs constitutionally express OPN1SW, RRH and OPN3 (Table 2). OPN1SW is a visual opsin, while the other two are non-visual opsins. Hence, RRH and OPN3 expression were selectively upregulated in migrated cells, but not in non-migrated cells (Fig. 7A and B), suggesting that only RRH and OPN3 are responsible for green LED irradiation-induced OFSC migration. RRH and OPN3, the two non-visual opsins in OFSCs, serve as the photoreceptors of green LED irradiation for the activation of ERK/MAPK/p38 signaling pathway during OFSC migration.

5. Conclusion

Green LED irradiation enhanced directional OFSC migration away from light source through activation of ERK signaling pathway. Increased ATP production facilitated kinase phosphorylation and the selectivity of phosphorylating on target kinases was governed by phototransduction mediated by RRH and OPN3. Pretreatment of OFSCs with green LED irradiation may serve a useful platform for future studies regarding wound repair using OFSCs.

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