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ORIGINAL ARTICLE

Induction of primitive pigment cell differentiation by visible light (helium–neon laser): a photoacceptor-specific response not replicable by UVB irradiation

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Abstract Solar lights encompass ultraviolet (UV), visible, and infrared spectrum. Most previous studies focused on the harmful UV effects, and the biologic effects of lights at other spectrums remained unclear. Recently, lights at visible region have been used for regenerative purposes. Using the process of vitiligo repigmentation as a research model, we focused on elucidating the pro-differentiation effects induced by visible light. We first showed that helium–neon (He–Ne) laser (632.8 nm) irradiation stimulated differentiation of primitive pigment cells, an effect not replicable by UVB treatment even at high and damaging doses. In addition, significant increases of mitochondrial DNA copy number and the regulatory genes for mitochondrial biogenesis were induced by He–Ne laser irradiation. Mechanistically, we demonstrated that He–Ne laser initiated mitochondrial retrograde signaling via a Ca^{2+}-dependent cascade. The impact on cytochrome *c* oxidase within the mitochondria is responsible for the efficacy of He–Ne laser in promoting melanoblast differentiation. Taken together, we propose that visible lights from the sun provide important environmental cues for the relatively quiescent stem or primitive cells to differentiate.

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In addition, our results also indicate that visible light may be used for regenerative medical purposes involving stem cells.

Keywords Helium–neon laser · Visible light · Melanoblast differentiation · Retrograde signaling · Mitochondrial biogenesis

Living organisms interact intimately with solar lights. Previous studies mostly focused on the damaging effects of lights at ultraviolet (UV) region. How visible light interacts with living organisms has remained unclear.

In the context of pigmentation, the effects of UV lights on differentiated epidermal and pigment cells have been well documented [1–3]. On the other hand, how lights interact with melanocyte precursor cells is not known. It has been shown that UV light imparts inhibitory effects on neural-crest-derived melanoblasts (MB) [4]. On the contrary, we demonstrated that low-energy visible light from helium–neon (He–Ne) laser (632.8 nm) imposes stimulatory effect on the same MB cells [5]. It is an intriguing phenomenon as lights at the UV region are generally considered to be biologically more active than lights at visible spectrum (400–700 nm).

He–Ne laser is a low-energy laser emitting monochromic red light. Clinically, low-energy He–Ne laser has therapeutic effects in various conditions including vitiligo [6, 7]. Vitiligo is a depigmentary disorder characterized by disappearance of functional melanocytes. In order for vitiligo skin to regain skin tone, the MB cells in the outer root sheath of the hair follicles serve as an important source for repigmentation [8]. In this process, the recovery from vitiligo is initiated by the activation and proliferation of MB cells, followed by their upward migration onto the nearby epidermis to form perifollicular pigment islands [9].

The molecular events explaining how He–Ne laser induces vitiligo repigmentation have not been fully elucidated despite reports describing the stimulatory events associated with red light treatment in the context of pigment cell migration and proliferation [5–7]. More specifically, cytochrome *c* oxidase in the mitochondria was reported to be the photoacceptor upon which He–Ne laser exerts its effect [10]. In addition, He–Ne laser has been shown to directly stimulate mitochondrial RNA and protein synthesis as well as increasing mitochondrial DNA replication [11, 12]. Moreover, cytosolic protein synthesis was also enhanced after He–Ne laser treatment [12, 13]. Recently, mitochondrial retrograde signaling has been proposed to be responsible for the cellular events induced by visible light [14]. Nevertheless, the mechanistic explanation for how He–Ne laser imparts pro-differentiating effects on MB cells remains obscure.

The major function of mitochondria is ATP synthesis. In addition, mitochondria play an important role in Ca^{2+} homeostasis and are the major intracellular source and immediate target of reactive oxygen species (ROS) [15]. Recently, we explored the alteration of mitochondrial activities during the stem cell differentiation and found a coordinated increase in mitochondrial biogenesis and the expression of antioxidant enzymes during the differentiation process of stem cells [16].

Since mitochondria contain the photoacceptor for visible light and are capable of initiating signaling to the nucleus, clarification of the mechanistic events involved in He–Ne laser-induced mitochondrial retrograde signaling will give important insights on how He–Ne laser induces MB cells to undergo differentiation. As the stem cells of MC lineage can be identified in the lower portion of mouse hair follicles throughout the hair cycle, the mouse MB cell lines were used for this study. More specifically, the NCCmelb4M5 cells are primitive cells belonging to mouse melanocyte lineage with negative expression for kit and tyrosinase [17]. The NCCmelb4 cells are immature MB cells with positive expression for kit but negative expression of tyrosinase [18]. The NCCmelan5 cells are differentiated MB cells with positive expression for both kit and typrosinase [19]. Using the primitive NCCmelb4M5 cells for evaluation of light-induced differentiation, the current study was launched to investigate the differential pro-differentiation effects of UVB and visible light on primitive MB cells.

Materials and methods

Culture of cells and He–Ne laser irradiation

Three melanoblast cell lines used were kindly provided by Professor Mizoguchi. The culture conditions were described in the Electronic supplementary material. The method for UV and He–Ne laser irradiation were described in our previous studies and in the Electronic supplementary material [20]. The viability of the cells after irradiation was evaluated using MTS (Cell-Titer 96 aqueous proliferation assay kit; Promega, Madison, WI) according to the manufacturer's instructions.

Detection of *KIT*, *tyrosinase-related protein-1*, and *DOPAchrome tautomerase* genes expression by real-time quantitative PCR

The cultured cells (5×10^5 cells) were irradiated with He–Ne laser or UVB and incubated for 24 h. After RNA extraction and amplification, detection of *KIT, tyrosinase-related protein-1* (*TYRP-1*) and *DOPAchrome tautomerase* (*DCT*) genes expressions were performed with an ABI Prism 7500 sequence detection system (Applied Biosys-

tems, New Jersey) and the primer pairs used are listed in Table 1 in the Electronic supplementary material.

Cytochrome *c* oxidase assay

After He–Ne laser irradiation, the cells were harvested and lysed immediately with an ice-cold radioimmunoprecipitation (RIPA) assay buffer. After centrifugation, the proteins were collected and quantified by the Bradford method (Bio-Rad Laboratories, Hercules, CA). The measurement of cytochrome *c* oxidase activity was determined according to the manufacturer's instruction (Sigma, St. Louis, MO).

Determination of intracellular ROS by flow cytometry

Cultured NCCmelb4M5 cells (1×10^6 cells) were seeded onto 6-well plate and pre-loaded with 10 μM of 2′,7′-dichlorodihydrofluorescein-diacetate (DCF-DA) solution (Sigma) for 30 min. The cells were then irradiated with He–Ne laser or 75 mJ/cm^2 UVB treatment. The cells treated with 500 μM H_2O_2 was used as a positive control. The detailed experimental procedures are described in the Electronic supplementary material.

Measurement of intracellular Ca^{2+} concentration

A 1.5×10^6/ml of NCCmelb4M5 cells were loaded with Fura-2-AM (Sigma) dissolved in DMSO for 30 min and irradiated with He–Ne laser. The cells were then washed with phosphate-buffered saline (PBS) and lysed with 0.1% Triton X-100. Fluorescence intensity of each lysate was measured with spectrophotometer (Hidex, Turku, Finland) by recording excitation signals at 340 nm and emission signal at 535 nm [21].

DNA extraction and the analysis of the mitochondrial DNA copy number

Cultured cells at a density of 5×10^5 cells/ml were irradiated with He–Ne laser and incubated for 24 h. The irradiated cells were resuspended in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.3) containing 1% SDS, 0.25 mg/ml RNase A, and 0.4 mg/ml proteinase K and incubated overnight. After repeated extraction with phenol/chloroform, DNA was precipitated with ice-cold 75% ethanol, air-dried, and dissolved in distilled water. The analysis of mtDNA copy number was measured by real-time PCR technique using the LightCycler FastStart DNA Master SYBR Green I (Roche, Mannheim, Germany) according to the manufacturer's instruction. The primer pairs used are listed, and the brief description of the assay is described in the Electronic supplementary material.

Analysis of the expression of mitochondrial biogenesis-related genes

The NCCmelb4M5 cells (1×10^7 cells) were irradiated with He–Ne laser and incubated for indicated intervals. The expressions of mitochondrial biogenesis-related genes including peroxisome proliferators-activated receptor γ coactivator-1α (PGC-1α), DNA polymerase γ (POLG), and mitochondrial transcription factor A (mtTFA) were performed by TaqMan-based real-time quantitative PCR. Total RNA was extracted with the TRI Reagent (Sigma) and chloroform, precipitated with isopropanol and dissolved in DEPC-H_2O. An aliquot of 1 μg RNA was reverse transcribed to cDNA with the Ready-to-Go RT-PCR kit (Amersham Biosciences, Uppsala, Sweden) and diluted 16 times for further quantitative PCR analysis. The experiment was performed with LightCycler TaqMan Master kit (Roche Applied Science) and primer pairs and probes number for specific genes expression were chosen according to the manufacturer's design. The information about the primer pairs and specific probes are listed in Table 1 in the Electronic supplementary material. The relative expression levels of target genes were normalized by the expression level of β-actin gene.

Flow cytometric analysis of kit expression levels

The NCCmelb4M5 cells were seeded onto 6-well plate at a density of 5×10^5 cells/ml and irradiated with He–Ne laser. After 24 h, the cells were collected and stained with anti-mouse CD117 (kit) equipped FITC antibody (1:100 dilution; eBioscience, San Diego, CA) and incubated for 30 min at 37°C. After washing twice with PBS, the cells were fixed with 1% paraformaldehyde in PBS and sorted by FACS (FACScan; Becton Dickinson, San Jose, CA, USA) for analysis (FL1 channel) by CELLQuest Prosoftware (Becton Dickinson). A specific Ca^{2+} chelator, 1,2-bis (*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA, Sigma) was used to clarify the role of Ca^{2+} levels on the kit expression of NCCmelb4M5 cells. After pre-treatment with BAPTA in cells for 1 h, the cells were irradiated with He–Ne laser and incubated for another 24 h. The treated cells were subjected to the flow cytometric analysis for the determination of kit expression.

Western blotting analysis

For determination of the phosphorylated cyclic AMP-response element binding protein (pCREB) expression, the NCCmelb4M5 cells (5×10^6) were seeded onto a 10-cm culture dish and irradiated with He–Ne laser. To examine the role of intracellular Ca^{2+} on pCREB expression, cells were pre-treated with BAPTA (Sigma) for 1 h. Thirty minutes

after He–Ne laser treatment, total cellular proteins were extracted by RIPA assay buffer. Details for Western blot analysis were described in the Electronic supplementary material.

Small interfering RNA experiment

The cultured NCCmelb4M5 cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The details on transfection were included in the Electronic supplementary material. The NCCmelb4M5 cells were then treated with He–Ne laser as described above.

Statistical analysis

The results are presented as means±SD of the results from at least three independent experiments. Statistical analysis was performed using unpaired Student's *t* test or one-way ANOVA with post hoc least significant test as appropriate. A *P* value of <0.05 was considered statistically significant.

Results

UVB irradiation reduced viability of NCCmelb4M5 cells UVB irradiation of 17 and 50 mJ/cm^2 significantly decreased the viability of NCCmelb4M5 cells to 82.3+9.8% and 62.3+5.9%, respectively, as compared with the sham-irradiated group while He–Ne laser treatment up to 2 J/cm^2 did not significantly affect cellular viability at 24 h after treatment.

He–Ne laser irradiation induced NCCmelb4M5 cells to express kit, a marker for differentiation, while UVB failed to do so, even at high and damaging irradiation doses Using NCCmelb4 cells, which intrinsically express kit, as a positive control, the kit expression of NCCmelb4M5 cells was determined after He–Ne laser and UVB treatment. After 2 J/cm^2 He–Ne laser treatment, the kit expression of NCCmelb4M5 cells showed approximately 2-fold increase at both mRNA and protein levels (Fig. 1a, b). In parallel with this finding, the mRNA expression of *DCT* significantly increased 1.95±0.53-fold after He–Ne laser treatment. However, the *TYRP-1* mRNA expression was not significantly altered by He–Ne laser. On the other hand, UVB treatment failed to induce kit expression of NCCmelb4M5 cells at both mRNA and protein levels even after 50 mJ/cm^2 irradiation, a dosage which induce significant cell damage as reflected by 40% reduced viability.

He–Ne laser induced functional development of NCCmelb4M5 cells Since our previous study showed that the differentiation of stem cells correlated with mitochondrial biogenesis

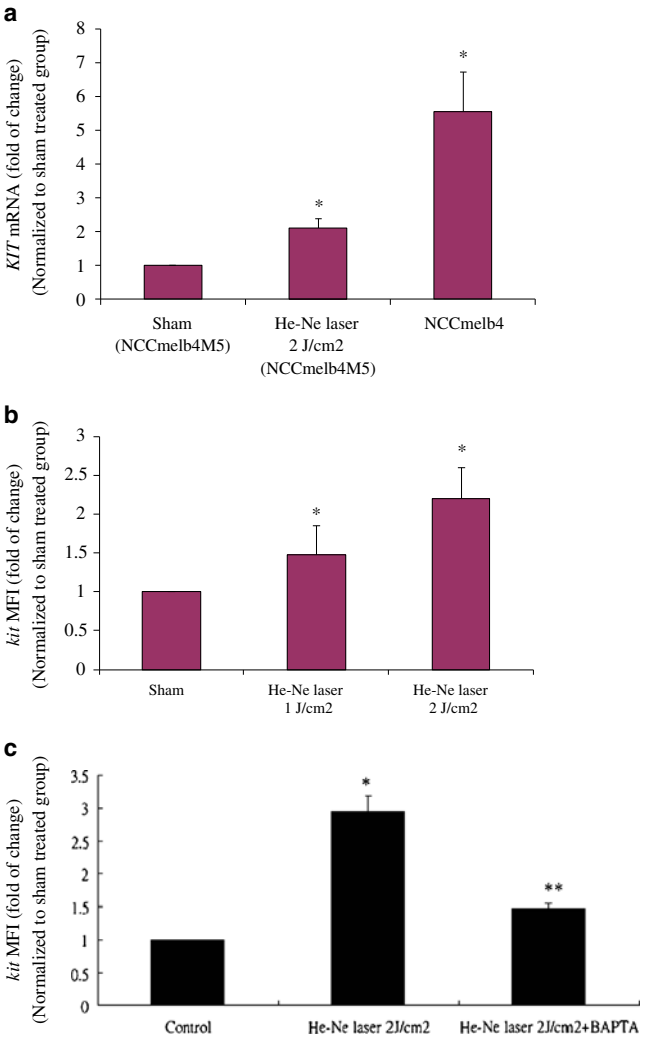

Fig. 1 He–Ne laser induced differentiation of NCCmelb4M5 cells 24 h after treatment. The expression of kit was considered as the quintessential marker of differentiation for NCCmelb4M5 cells. **a** He–Ne laser irradiation induced *KIT* expression at mRNA level on NCCmelb4M5 cells as determined by quantitative PCR. The *KIT* expression of the untreated NCCmelb4 cells, which intrinsically express *KIT*, served as the positive control. **b** By flow cytometry, the protein expression of kit on NCCmelb4M5 cells after irradiation with He–Ne laser were determined. **c** Pre-treating NCCmelb4M5 cells with Ca^{2+}-chelator BAPTA abrogated the effect of He–Ne laser on inducing kit expression as revealed by flow cytometry. *p<0.05 as compared with control; **p<0.05 as compared with the He–Ne laser-treated group

[16], the mtDNA copy number of the melanoblast cells at various differentiation stages was evaluated. The mtDNA copy number was highest in the most-differentiated melanoblast NCCmelan5 cells, followed by the less-differentiated NCCmelb4 cells. The most primitive NCCmelb4M5 cells had the lowest copy number of mtDNA (Fig. 2a). These results corroborated with our previous findings [16]. In addition, we also found that at 24 h after He–Ne laser treatment, the mtDNA copy number

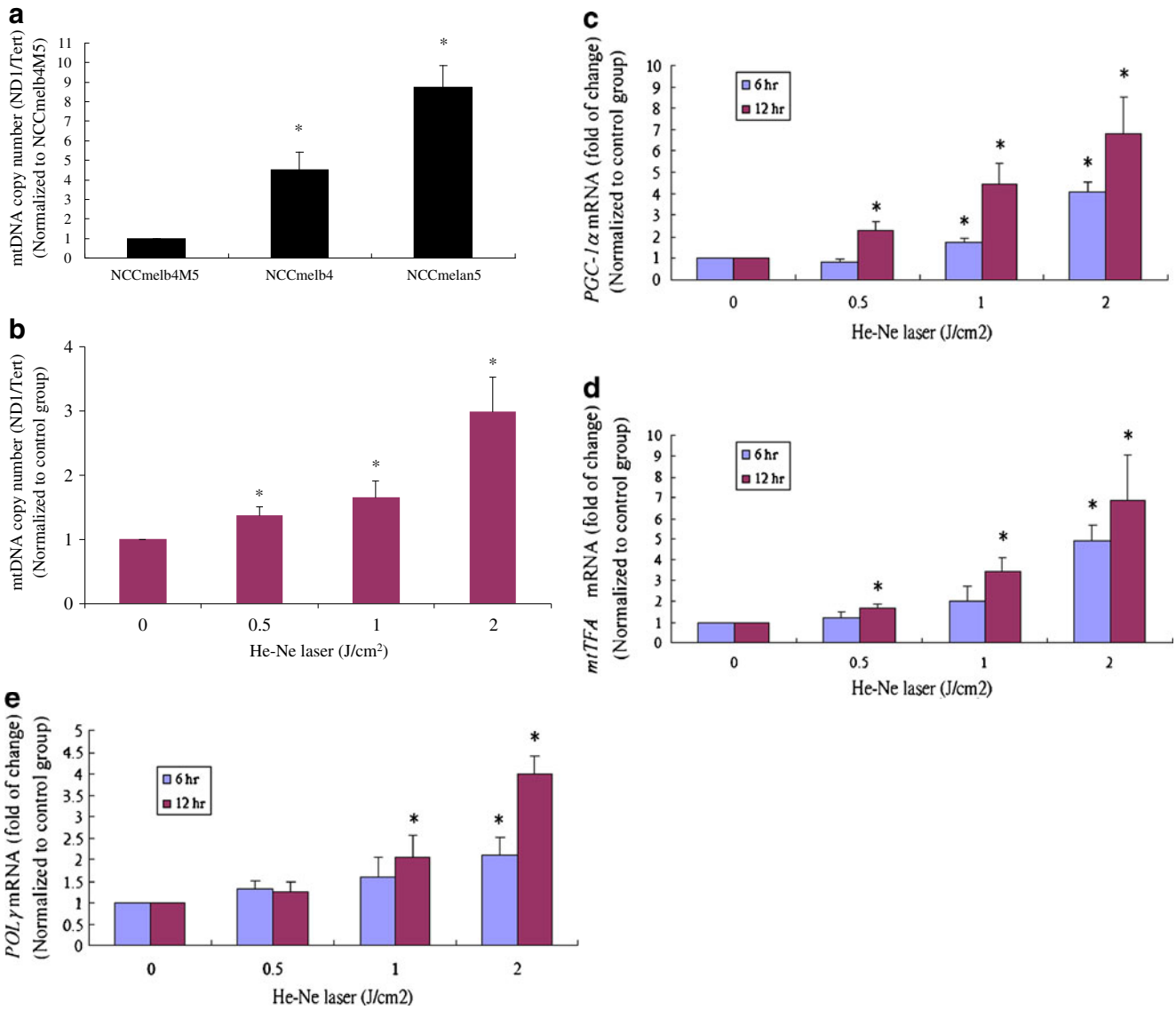

Fig. 2 Increased mitochondrial DNA (*mtDNA*) copy number correlated with differentiation status of melanoblast cells and He–Ne laser increased the expressions of mitochondrial biogenesis-associated genes in time- and dose-dependent manners. **a** The mtDNA copy number was highest in the most-differentiated melanoblast NCCmelan5 cells, followed by the less-differentiated NCCmelb4 cells. The most primitive NCCmelb4M5 cells had the lowest copy number of mtDNA. **b** At 24 h after He–Ne laser irradiation, a dose-dependent increase in mtDNA copy number was observed in NCCmelb4M5 cells. The expression of mitochondrial biogenesis-associated genes including **c** *PGC-1α*, **d** *mtTFA*, and **e** *POLγ* in NCCmelb4m5 cells showed a time- and dose-dependent increased at indicated earlier time intervals. *$p<0.05$

of NCCmelb4M5 cells showed a dose-dependent increase of approximately 3-fold after irradiation at 2 J/cm^2 (Fig. 2b). This finding further strengthened and validated the notion that He–Ne laser irradiation imparted a pro-differentiation effect on NCCmelb4M5 cells.

He–Ne laser stimulated mitochondrial biogenesis of NCCmelb4M5 cells As He–Ne laser irradiation increased the mtDNA copy number of NCCmelb4M5 cells, we next evaluated the expressions of the crucial genes associated with mitochondrial biogenesis including *PGC-1α, mtTFA*, and *POLγ* at indicated time points after He–Ne laser irradiation. Using quantitative PCR, we demonstrated that the mRNA expressions of these genes showed time- and dose-dependent increase after He–Ne laser treatments (Fig. 2c–e). These results indicated that He–Ne laser induced mitochondrial biogenesis, a process that occurred with induction of kit expression on NCCmelb4M5 in a coordinated manner.

Immediate cellular responses induced by He–Ne laser After we confirmed that He–Ne laser induced functional devel-

opment and stimulated mitochondrial biogenesis of NCCmelb4M5 cells, we explored the immediate events after He–Ne laser treatment. Increases in intracellular Ca^{2+} and ROS levels have been proposed as possible initiating events associated with He–Ne laser irradiation [14]. As demonstrated in Fig. 3, a more than 2-fold increase in cytochrome *c* oxidase activity was observed immediately after He–Ne laser treatment. This event was accompanied by an approximately 3-fold increase in intracellular Ca^{2+} levels, indicating that mitochondria-related and Ca^{2+}-dependent signaling pathways play initiating roles after He–Ne laser treatment. On the other hand, the levels of ROS were not significantly elevated after He–Ne laser treatment while UVB induced immediate ROS elevation (Fig. 4). These finding collaborated with our previous study on He–Ne laser [22]. Since we had shown that He–Ne laser significantly increased the pCREB expression of

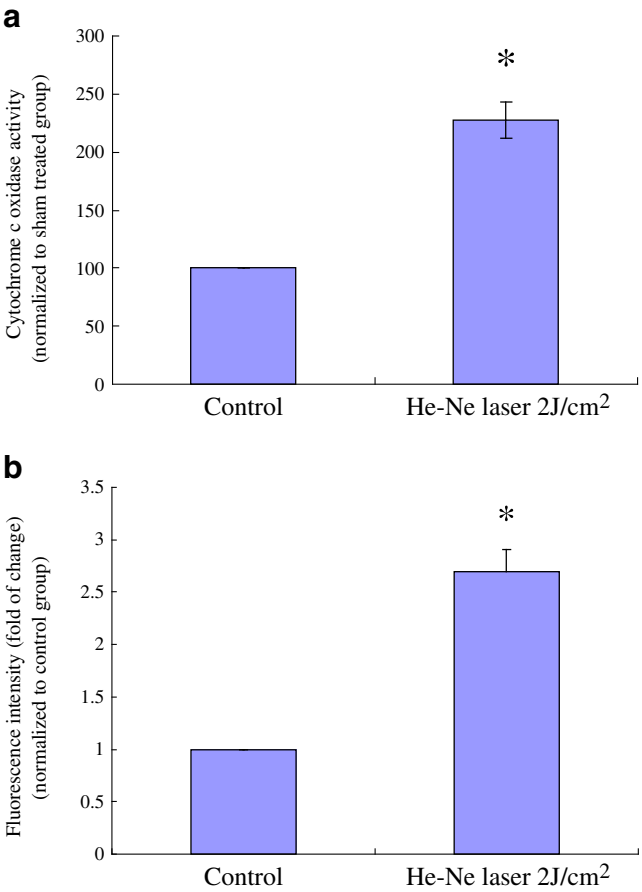

Fig. 3 He–Ne laser irradiated NCCmelb4M5 cells showed immediate increase of cytochrome *c* oxidase activity and intracellular Ca^{2+} levels. **a** The activity of cytochrome *c* oxidase (mitochondrial Complex IV) was assayed by spectrophotometry according to the method described immediately after He–Ne laser treatment. The data are expressed as relative levels of cytochrome *c* oxidase activity compared with the sham-irradiated group taken as 100%. **b** By flow cytometry, the intracellular Ca^{2+} levels in NCCmelb4M5 cells were determined with Fura-2-AM. *$p<0.05$

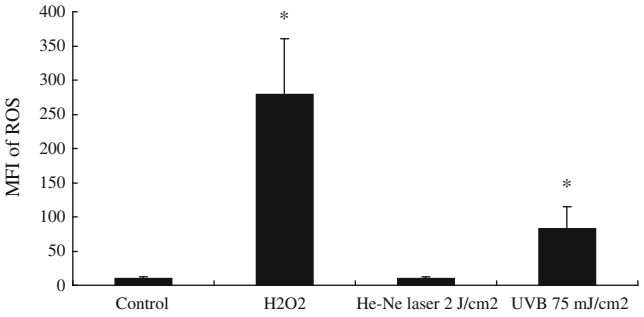

Fig. 4 He–Ne laser did not significantly increase the intracellular ROS levels. Intracellular ROS levels were evaluated with the probe of DCF-DA by flow cytometry. Using exogenous H_2O_2 treatment on NCCmelb4M5 cells as a positive control, the intracellular levels of ROS in NCCmelb4M5 cells after He–Ne laser irradiation did not significantly increase while UVB irradiation induced significant ROS formation. *$p<0.05$ compared with control group

melanocytes [7] and it was known that intracellular Ca^{2+} levels modulate pCREB expressions [23], we next determined the relationship between these two molecular events. As demonstrated in Fig. 5, He–Ne laser irradiation significantly upregulated the pCREB expression. Pre-treating cells with Ca^{2+} chelating agent BAPTA significantly abrogated the induction effect of He–Ne laser on pCREB expression. Subsequently, we investigated whether similar treatment would also abrogate the pro-differentiation effect of He–Ne laser on NCCmelb4M5 cells. As shown in Fig. 1c, BAPTA treatment also abrogated the He–Ne laser-induced kit expression, indicating that the signaling pathways induced by He–Ne laser occurred in a Ca^{2+}-dependent manner. To confirm the functional role of CREB cascade on primitive melanoblast cell development, gene silencing of CREB signaling by small interfering RNA (siRNA) approach was used. Accordingly, NCCmelb4M5 cells with CREB knockdown via siRNA showed no induction of kit expression after He–Ne laser treatment (0.93+0.11-fold of normal control) while the negative control of siRNA showed kit induction after He–Ne laser treatment (1.68+0.47-fold of normal control). In parallel with this finding, the enhanced *DCT* mRNA expressions induced by He–Ne laser were also abrogated by this siRNA approach.

Discussion

In this study, we demonstrated that He–Ne laser induced differentiation and enhanced mitochondrial biogenesis of the primitive NCCmelb4M5 cells via a Ca^{2+}-dependent mitochondrial retrograde signaling. Previously, we had shown that He–Ne laser is able to stimulate immature melanoblast migration, a biological characteristic of MB

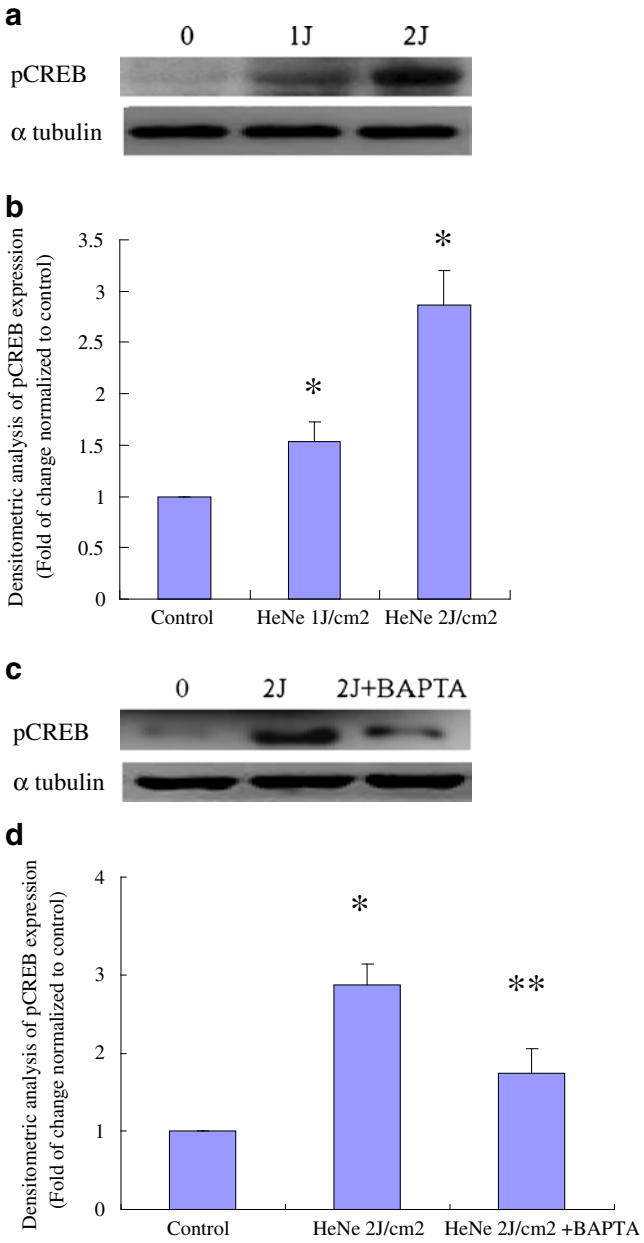

Fig. 5 He–Ne laser stimulated the expression of phosphorylated CREB via a Ca^{2+}-dependent manner. **a** By Western blot analysis, the levels of phosphorylated CREB (*pCREB*) on NCCmelb4M5 cells increased dose dependently after He–Ne laser treatment. **b** Densitometric analysis of (**a**), the ratios of pCREB to α-tubulin expressions of different treatment groups normalized to the control group. **c** Pretreating NCCmelb4M5 cells with Ca^{2+}-chelator BAPTA abrogated the effect of He–Ne laser on enhancing pCREB expression. **d** Densitometric analysis of (**c**), the ratios of pCREB to α-tubulin expressions of different treatment groups normalized to the control group. *$p<0.05$ as compared with control; **$p<0.05$ as compared with the He–Ne laser-treated group

undergoing differentiation [5]. Intriguingly, UV light, which is known for its potent photobiological actions, was not able to induce similar effect [4]. In support with the previous report, the current study demonstrated that UVB was not able to induce differentiation of NCCmelb4M5 cells. In order for lights to elicit biological effect, the signaling between photoacceptor and its downstream cascade is of utmost importance. It has been shown that mitochondria-related signaling pathway is responsible for the He–Ne laser-induced biological effects [7, 22]. As demonstrated in this study, one of the earliest events induced by He–Ne laser involves immediate increased activities of cytochrome *c* oxidase and elevated intracellular Ca^{2+} levels. The increased intracellular Ca^{2+} level was known to stimulate mitochondrial gene expression through signaling related to CREB [23]. In terms of its impact on pigment cells, CREB binds to cyclic AMP-response element present in the M promoter of the *Mitf* gene which subsequently leads to the up-regulations of key regulatory genes required for pigment cell development [24]. In terms of its role on mitochondrial regulation, CREB is known to modulate PGC-1α [25], the master regulator of mitochondrial biogenesis [26] which in turn induces the expression of mtTFA, a specific mitochondrial DNA binding protein that serves essential role in mitochondrial transcription and mtDNA replication [27]. Therefore, as He–Ne laser irradiation was received by cytochrome *c* oxidase in the mitochondria, an increased intracellular Ca^{2+} level was induced. This event increased the expression of pCREB, which plays an essential role in differentiation and mitochondrial biogenesis of MB cells. Pre-treatment with Ca^{2+} chelating agent BAPTA abrogated the enhanced expression of pCREB and the induction of kit on NCCmelb4M5 cells. These results confirmed that the alterations induced by He–Ne laser irradiation occurred in a Ca^{2+}-dependent manner. It should be noted that although the enhanced cytochrome *c* oxidase activity may also result from increased mitochondrial biogenesis, it is unlikely to represent the situation encountered in this study since cytochrome *c* oxidase activity was enhanced immediately after He–Ne laser irradiation while increased mitochondrial biogenesis was observed at later time points. A schematic diagram summarizing our findings is shown in Fig. 6.

Since cytochrome *c* oxidase is located within the mitochondria, the molecular events we had just unraveled clearly exemplified a mitochondrial retrograde signaling. Conventionally, the transfer of information is initiated from the nucleus and cytoplasm to mitochondria. On the contrary, the retrograde signaling originates due to the alterations in the functional states of mitochondria, which then culminate in alterations of nuclear gene expression [28]. The concept that light stimulation may invoke mitochondrial retrograde signaling in mammalian cells was proposed as early as 1988. In rat mitochondria, it was shown that He–Ne laser irradiation increased ADP/ATP exchange [29]. Subsequently, Pastore et al. showed that He–Ne laser induced activation of purified cytochrome *c*

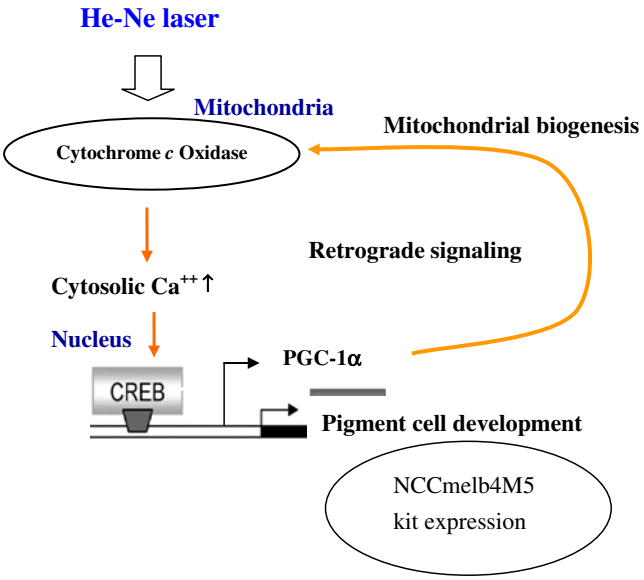

Fig. 6 A schematic diagram summarizing the proposed mitochondrial retrograde signaling involved in He–Ne laser-induced NCCmelb4M5 cell differentiation is shown. He–Ne laser irradiation stimulated cytochrome *c* oxidase activity in NCCmelb4M5 cells, which resulted in increased intracellular Ca^{2+} levels and subsequent enhanced pCREB expression. The increased expressions of pCREB ultimately resulted in increased mitochondrial biogenesis and NCCmeb4M5 differentiation. The specific photoacceptor of He–Ne laser within the mitochondria provided a reasonable explanation for superior efficacy of He–Ne laser on inducing NCCmelb4M5 cells differentiation as compared with UV lights

oxidase [10]. Cytochrome *c* oxidase, the identified photoacceptor for red light irradiation, is the terminal enzyme of the respiratory chain in eukaryotic cells, which mediates the transfer of electrons from cytochrome *c* to molecular oxygen. It is a large membrane protein which contains two heme moieties, two redox-active copper sites, one zinc, and one magnesium ion as the potential chromophores for visible lights. The photochemical and photobiolgoical consequences that might result from the absorbance of He–Ne laser irradiation by cytochrome *c* oxidase remains unclear. Different intermediary events have been proposed including generation of ROS and changes in Ca^{2+} flow to initiate the mitochondrial retrograde signaling. Under our experimental conditions, however, ROS production is unlikely a key mediator as its level was not altered by He–Ne laser treatment. On the other hand, rapid increase of intracellular Ca^{2+} levels suggested that alterations of Ca^{2+} flow to be an important intermediary event after cytochrome *c* oxidase stimulation. Mitochondria play a crucial role in Ca^{2+} storage and homeostasis in mammalian cells. In addition, mitochondrial stress was associated with elevated intracellular Ca^{2+} levels that resulted in altered cell physiology and gene expressions [30]. In addition, using cells with mutated mitochondrial tRNA, Arnould et al. [31] showed that the elevated cytosolic Ca^{2+}-induced activation of calcium/calmodulin kinase IV, which in turn activated CREB signaling. Addition of specific chelators to remove free Ca^{2+} abrogated the activation of various factors and induced transcription of nuclear target genes, confirming the role of Ca^{2+} in mitochondrial retrograde signaling. Corroborating with these previous reports, MB cell differentiation induced by He–Ne laser also involves Ca^{2+}-dependent pCREB-mediated mitochondrial retrograde signaling.

Although UVB has been demonstrated to increase intracellular Ca^{2+} levels in immortalized keratinocytes [32], it is obvious that UVB irradiation lacked specific photoacceptor as both DNA and tryptophan can serve as photoacceptors for UVB [33]. Moreover, the cellular damages induced by UVB irradiation is far greater than He–Ne laser treatment as more than 50 mJ/cm^2 UVB irradiation significantly reduced the NCCmelb4M5 cell viability while He–Ne laser treatment at 2 J/cm^2 did not alter the growth NCCmelb4M5 cells. The differences in terms of photoacceptor specificity provide a reasonable explanation for the differential effects of UVB and visible light on MB development.

One limitation of this study is that the experimental results were derived from immature melanoblast cell line. Further validation with primary cells would strengthen the findings demonstrated in this study. Unfortunately, primary cells are not currently available at our facility for experimentation. It is possible that since NCCmelb4M5 cells was established by an single-cell cloning method, the cells derived may have some subtle differences when compared with primary cell culture, which may be more heterogeneous in nature. However, our previous studies using primary melanocytes have shown that He–Ne laser increased pCREB expression, a finding that was also documented in this study [7]. In addition, previous study has shown that similar effect of bone morphogenetic protein on induction of kit expression in both NCCmelb4M5 cells and primary cultures of neural crest cells [34]. Therefore, we expect the effects of He–Ne laser on primary cells to be very similar to the cell line used in this study. It should be noted that although He–Ne laser increased the *DCT* mRNA expression, the mRNA expression of *TYRP-1* was not significantly altered by similar treatment. One reasonable explanation is that a distinct regulatory mechanism may exist for *DCT* during early stages of development since *DCT* gene is expressed early in pigment cell development, prior to other members of the *tyrosinase* gene family including *TYPR-1* [35].

In summary, we have clarified the molecular mechanisms involved in the He–Ne laser-induced pro-differentiation effects of primitive MB cells, an event not replicable by the more intense UV irradiation. Although UV light is generally considered more intense and potent than visible light, the

specific photoacceptor of He–Ne laser light in the mitochondria and the Ca^{2+}-mediated mitochondrial retrograde signaling are responsible for He–Ne laser's effective pro-differentiation effect on MB cells. Since solar radiation encompasses lights at both UV and visible spectra, we propose that visible lights from the sun provide important environmental cues for the relatively quiescent stem or primitive cells to differentiate and to counteract the harmful impacts from UV lights. In addition, modulation of primitive stem cells may be accountable for the therapeutic effects of visible light in the context of regenerative medicine.

Conflict of interest The authors state no conflict of interest.

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