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ORIGINAL ARTICLE

Photobiomodulation promotes adenoviral gene transduction in auditory cells

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Abstract

Gene therapy is the delivery of a therapeutic gene into target cells to treat disorders by replacing disease-causing mutated genes with healthy ones. Gene therapy of the inner ear has been recently described, with applications for sensorineural hearing loss. However, gene delivery to the location of the inner ear, and thus efficacy of therapy, is challenging. Photobiomodulation (PBM) with a low-level laser has been suggested to have a therapeutic effect and has the potential to augment gene therapy. To investigate whether PBM improves the rate of adenovirus (Ad)-mediated viral delivery, we compared low-level laser therapy (LLLT) and non-LLLT HEI-OC1 cells treated with an Ad viral vector carrying green fluorescent protein (GFP). Cultured HEI-OC1 cells were divided into six groups: no treatment control, LLLT only, 1 μL Ad-GFP, 3 μL Ad-GFP, 1 μL Ad-GFP + LLLT, and 3 μL Ad-GFP + LLLT (LLLT: 808 nm at 15 mW for 15 min). Cells were irradiated twice: at 2 h and again at 24 h. A nonparametric Mann-Whitney *U* test was used to statistically analyze differences between the control and treatment groups. The viral inoculations used in this study did not change the amount of viable HEI-OC1 cells ($N = 4–8$). The 1 μL Ad-GFP + LLLT and 3 μL Ad-GFP + LLLT groups showed an increased density of GFP-positive cells compared to 1 μL and 3 μL Ad-GFP cells ($N = 5–8$, 1 μL: $p = 0.0159$; 3 μL: $p = 0.0168$,). The quantitative analysis of the epifluorescence of the 1 μL Ad-GFP + LLLT, and 3 μL Ad-GFP + LLLT groups revealed increased GFP expression/cell compared to 1 μL and 3 μL Ad-GFP cells ($N = 6–15$, 1 μL: $p = 0.0082$; 3 μL: $p = 0.0012$). The RT-qPCR results were consistent ($N = 4–5$, $p = 0.0159$). These findings suggest that PBM may enhance the gene delivery of Ad-mediated viral transduction, and the combination of the two may be a promising tool for gene therapy for sensorineural hearing loss.

Keywords Auditory cell · Gene therapy · Hearing loss · Photobiomodulation

Introduction

Gene therapy is the delivery of a therapeutic gene into target cells to treat disorders. In this method, disease-causing mutated genes are replaced with healthy ones, thereby inactivating the malfunctional mutated gene or by introducing a gene that fights disease [1, 2]. Gene therapy can be divided into two categories: germline gene therapy and somatic gene therapy. Somatic gene therapy is more widely used because it only affects the treated organ of an individual [3]. Many current studies have shown the beneficial effects of gene therapy, most of which have focused on cancer [4], but few have been devoted to neurological and ocular diseases [5, 6].

Gene therapy for the inner ear may be used as a therapeutic tool for sensorineural hearing loss, a condition caused by hereditary hearing loss, ototoxicity, and acoustic overexposure [7–9]. However, there are some limitations to this therapy due to the location and structure of the inner ear. The cochlea is located deep inside the skull and is surrounded by complex structures that make delivery of the gene or other therapeutic agents difficult. Additionally, the cochlea is divided into three compartments and harbors two different fluid compositions.

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Thus, mechanical disruption of the barrier between these compartments caused by the delivery of therapeutic agents results in a disturbance of cochlear homeostasis [10, 11]. Therefore, achieving higher gene transduction to target cells without harming the resident cells has always been challenging.

There are two major gene delivery systems: non-viral systems, which use mediators such as cationic liposomes, electroporation, and gene guns; and viral vector systems, which use DNA or RNA viruses. Regrettably, non-viral gene mediators can cause considerable damage to the cell membrane and decrease the effectiveness of the transduction process. In contrast, viral vector-mediated gene transfer is a more efficient means of delivery, and the most commonly used viral vector gene therapy is adenovirus (Ad) [12, 13].

Photobiomodulation (PBM) is the application of laser therapy using a relatively low-level laser (1–500 mW) at wavelengths between 600 and 1000 nm [14–17]. PBM is clinically offered as a unique treatment option for wound healing, pain relief, anti-inflammation, anti-edema, and nerve regeneration [18–23]. PBM also promotes stem cell proliferation and modulation of cellular metabolic processes [23–25]. Previous reports have shown that laser application itself can also be used as a gene transduction technique [26–28]. Considering these positive effects of PBM, we investigated the effects of PBM on the transduction of gene therapy of the inner ear.

We proposed a null hypothesis that the Ad transduction process causes cytotoxicity in auditory cell lines and does not affect gene delivery. We investigated whether the Ad transduction process is toxic to the auditory cell line (HEI-OC1) and evaluated whether PBM improves Ad-mediated viral gene transfer in HEI-OC1 cells.

Materials and methods

Cell culture

The House Ear Institute-Organ of Corti 1 (HEI-OC1) mouse auditory cell line was cultured continuously in high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Corning, Tewksbury, MA, USA) supplemented with 10% (*v*/*v*) heat-inactivated fetal bovine serum (FBS; Equitech-Bio, Kerrville, TX, USA) and maintained at 33 °C in a humidified incubator under 5% CO_2 (Thermo Scientific) and non-permissive conditions. Cells were divided into six groups: no treatment control, laser only, 1 μL/mL Adenovirus-green fluorescent protein (Ad-GFP), 3 μL/mL Ad-GFP, 1 μL/mL Ad-GFP + laser, and 3 μL/mL Ad-GFP + laser. The Ad-GRP condition refers to an Ad vector carrying the GFP gene. After cells were grown to confluence, the media was replaced with fresh media in the presence or absence of Ad-GFP.

Ad-GFP viral inoculation

Replication-incompetent adenovirus-GFP (6.6×10^8 OPU/mL) was kindly donated by Prof. Yong-Ho Park (Chungnam National University). Ad-GFP vectors, at concentrations of 1 μL/mL and 3 μL/mL, were inoculated into 96- and 24-well plates (SPL, Pocheon, Gyeonggi, Korea) containing HEI-OC1 cells at a density of 1×10^3 cells/mL and incubated at 33 °C under 5% CO_2 for 24 h. After the infection process (Ad-GFP for 24 h), the samples were replaced with fresh media supplemented with 10% (*v*/v) heat-inactivated FBS (Equitech-Bio) and cultured for 5 days. Experimental procedures are illustrated in Fig. 1.

Cell viability of HEI-OC1 cells treated with Ad-GFP

To determine whether viral vector transduction is toxic to auditory cells, cell viability was observed by differential interference contrast microscopy (Olympus DP27, Hachioji-shi, Tokyo, Japan) and assessed at days 1, 3, and 5 after Ad-GFP infection using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (MTT: Sigma-Aldrich, St. Louis, MO, USA) assay. For the assay, 50 μL MTT solution (2 mg/mL) was added to each well and plates were incubated for 4 h at 33 °C under 5% CO_2. The medium in each well was removed and purple formazan crystals synthesized by the cells were dissolved in 150 μL dimethylsulfoxide (Junsei Chemical Co. Ltd., Tokyo, Chuo-Ku, Japan) per well. After 20 s of plate agitation (shaking incubator: Amersham, Buckinghamshire, HP7 9NA, UK), the optical density (OD) was measured on a microplate reader (Asys UVM340, Biochrom Ltd., Cambridge, UK) at a wavelength of 540 nm. Cell viability was calculated using the following formula:

$$\text{Cell viability } (\%) = \text{Mean OD in treated wells} \div \text{Mean OD in control wells} \times 100.$$

Laser irradiation

In this study, a near-infrared diode laser (WonTech, Daejeon, Chungnam, Korea) with a wavelength of 808 nm was used to irradiate the Ad-GFP plus laser and laser only samples. The target monolayer of cells was directly irradiated at an intensity of 15 mW for 15 min (total energy density = 13.5 J/cm^2) at 2 h and 1 day after Ad-GFP treatment. The power of the laser was measured at the bottom of the empty plate before actual laser irradiation to the cell with a SOLO2 laser power meter (Gentec-EO, G2E 5N7, Quebec, Canada) and an XLP12-1S-H2-DO detector head (Gentec-EO, G2E 5 N7). Table 1 lists specific laser parameters.

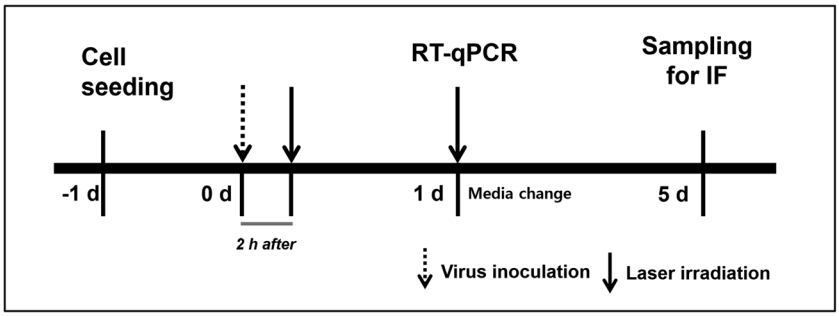

Fig. 1 Schematic of the experimental procedure. The auditory cell line was seeded and incubated first for 24 h, then infected with viral vectors for another 24 h. Photobiomodulation with a laser was performed twice, at 2 h and 1 day post transduction. Viral vector expression efficiency was evaluated by RT-qPCR on day 1 after second irradiation and by immunofluorescence (IF) on day 5

Epifluorescence analysis

For epifluorescence analysis, cells were rinsed twice with 1× phosphate-buffered saline (PBS, Bio-Rad, Hercules, CA, USA) and fixed with 4% paraformaldehyde (Alfa Aesar, Ward Hill, MA, USA) in PBS for 15 min at room temperature (RT) and washed twice with 1× cold PBS. Blocking was performed in PBS containing 10% normal goat serum (Vector Laboratories, Burlingame, CA, USA) and 0.3% Triton X-100 (Sigma-Aldrich) for 30 min at RT. Subsequently, cells were washed three times with 1X PBS for 5 min each. Samples were incubated with Myosin VIIa (MyoVIIa) which is a protein that is encoded by the *MYO7A* gene and affected to profoundly hearing function. The primary antibody (1:500 dilution) (rabbit MyoVIIa, Proteus Biosciences, Ramona, CA, USA) in blocking solution, which was composed of 1% bovine serum albumin (BSA: Santa Cruz Biotechnology, Dallas, TX, USA) and 10% normal goat serum in PBS (pH 7.4) overnight at 4 °C. Samples were washed three times with 1× PBS each after primary antibody incubation. Next, the secondary antibody (1:500) (Alexa Fluor 610-conjugated goat anti-rabbit IgG, Invitrogen, Waltham, MA, USA) in blocking solution was labeled for 1 h. Samples were then washed three times with cold 1× PBS. Finally, cells were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories). Cells were visualized under a confocal microscope (Olympus FW3000, Hachioji-shi, Tokyo, Japan) and representative images were captured for cell density calculations. In the acquired images, GFP-positive cells were counted. For the measurement of HEI-OC1 cell density, all nuclei stained by DAPI within the designated area (200 × 200 μm) was counted. GFP expression within the region of interest was calculated (determined by a white line drawn manually by the author). All cell counting and GFP expression measurements were done using ImageJ software 1.43u (National Institute of Mental Health, Bethesda, MD, USA).

Table 1 Laser information and parameters

Manufacturer	Wontech Co. Ltd., KOR
Light type	Diode laser
Number of emitters	1
Mode	Continuous wave (CW)
Wavelength	808 nm
Intensity	15 mW
Irradiation time	15 min
Total energy density	13.5 J/cm^2

RT-qPCR analysis for GFP gene expression in Ad virus-transfected cells

Total RNA was isolated using the GeneAll Hybrid-R™ Kit (GeneAll Biotechnology, Sougpa, Seoul, Korea) following the manufacturer's protocol. cDNA was synthesized using forward and reverse primers (Oligomer; Bioneer, Daejeon, Chungnam, Korea). GFP primer sequences are as follows: forward primer, 5'-CGACGTAAACGGCCACAAGT-3′ and reverse primer, 5'-TCGTGCTGCTTCATGTGGTC-3′. GFP expression was measured on an Applied Biosystems-7500 Real-Time PCR System (Life Technologies, Carlsbad, CA, USA).

Statistical analysis

The data was statistically analyzed by GraphPad Prism (GraphPad Software, La Jolla, CA, USA) or SPSS (IBM SPSS statistics, Armonk, NY, USA) software. A Kolmogorov–Smirnov test was used to determine whether the data was parametric or non-parametric. Significant differences between the control and treatment groups were statistically analyzed using a *t* test in case of a parametric distribution and Mann-Whitney *U* test in case of a nonparametric distribution. To reject the null hypothesis, the estimation of the power analysis was checked by determining the probability of type II errors (beta value). A p value less than 0.05 was considered statistically significant. In the figures, p values are defined as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Results

Viral inoculation does not alter the number of viable HEI-OC1 cells

To determine whether Ad transduction into HEI-OC1 cells causes cell damage, cells were transfected with 1 or 3 μL/mL Ad vector carrying the GFP gene, and cell viability was analyzed by the MTT assay 5 days after inoculation (Fig. 2). HEI-OC1 cells were not damaged under either condition relative to the control group. Light microscopy revealed no difference in the number of viable cells between groups on day 5 (Fig. 2a). The MTT assay revealed no difference in cell viability between groups (Fig. 2b). These results suggest that the use of 1 or 3 μL/mL Ad vector is not toxic to HEI-OC1 cells 5 days after transfection. All subsequent experiments were carried out using these two concentrations.

PBM enhances the Ad-mediated immunofluorescence for GFP expression in HEI-OC1 cells

To analyze the biological characteristics of the cell line and viral vector transduction, epifluorescence analysis was performed using a MyoVIIa antibody and self-expressed GFP from transduced cells. The MyoVIIa antibody was chosen as MyoVIIa is a unique feature of cochlear and vestibular hair cells. All HEI-OC1 cells used in this study were MyoVIIa-positive, which indicate that the cells represent biological characteristics of cochlear and vestibular hair cells. After viral vector inoculation, transduced cells expressed GFP, which was observed as a fluorescent green color with an epifluorescence microscope. GFP expression was not observed in either the control or laser only groups but was observed in all viral vector-treated groups. However, not all cells within the viral vector-treated groups expressed GFP. In addition, there was a significant difference in the number of GFP-expressing cells among viral vector-treated groups. The number of GFP-positive cells revealed a direct viral vector dose-dependent relationship, that was further enhanced by exposure of the PBM laser. Of the viral vector-treated conditions, the density of GFP-positive cells was higher in the laser-treated group than the non-laser group (upper row) (Fig. 3a). Images acquired from epifluorescence analysis were used to quantify the number of GFP-positive cells. The number of GFP-positive cells was 66.6 ± 15.7 and 87.3 ± 35.7 in the 1 μL/mL and 3 μL/mL virus-treated groups without laser application, respectively (Fig. 3b). Similarly, the number of GFP-positive cells was 99.0 ± 19.2 and 150.3 ± 39.4 for 1 μL/mL virus + laser and 3 μL/mL virus + laser groups, respectively. A significant increase in the number of GFP-positive cells was observed after PBM treatment (Fig. 3b) (1 μL/mL virus + laser; observational power—0.674, β value—0.329, $p = 0.0159$; 3 μL/mL virus + laser; observational power—0.788, β value—0.212, $p = 0.0168$). This result suggests that PBM increases the transduction rate of genes into auditory cells using viral vectors. Density of the HEI-OC1 cells did not change after LLLT exposure (Man-Whitney U test, $U = 30.50$, $p = 0.9577$, Fig. 1c). This result indirectly suggests that

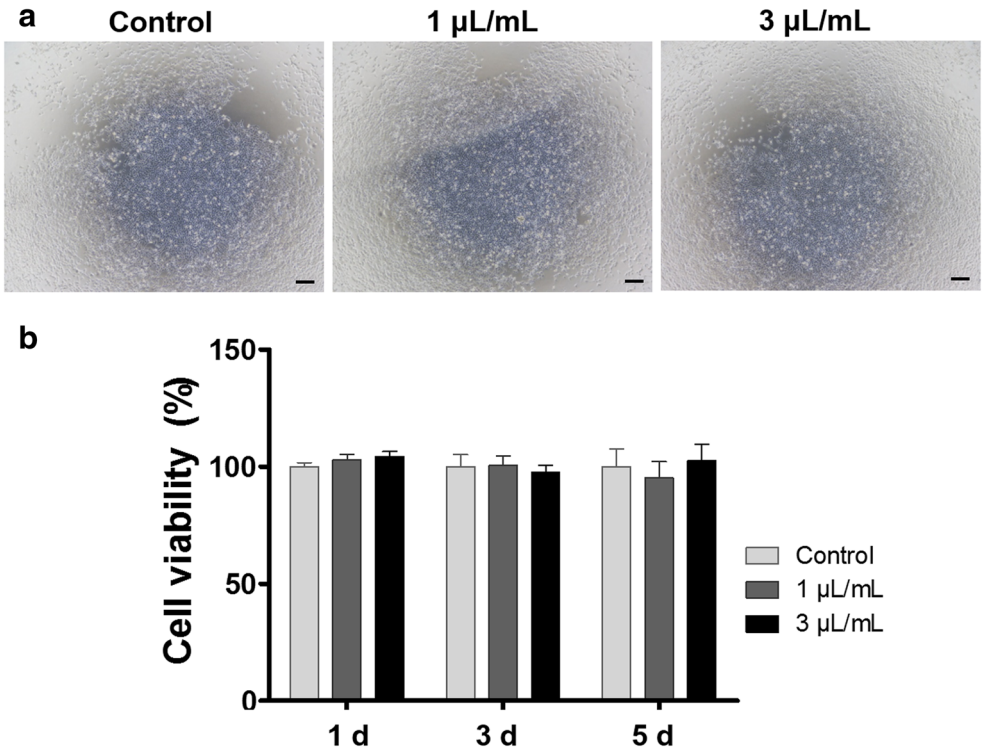

Fig. 2 Cell viability of HEI-OC1 cells after Ad-GFP inoculation. **a** Representative images of auditory cells 5 days after Ad-GFP inoculation obtained by differential interference contrast microscopy showing no difference in cell density among groups (scale bar 200 μm). **b** Histogram representing cell viability percentage measured by the MTT assay over time. There was no difference in cell viability after viral inoculation of Ad-GFP (1 μL/mL and 3 μL/mL on day 5)

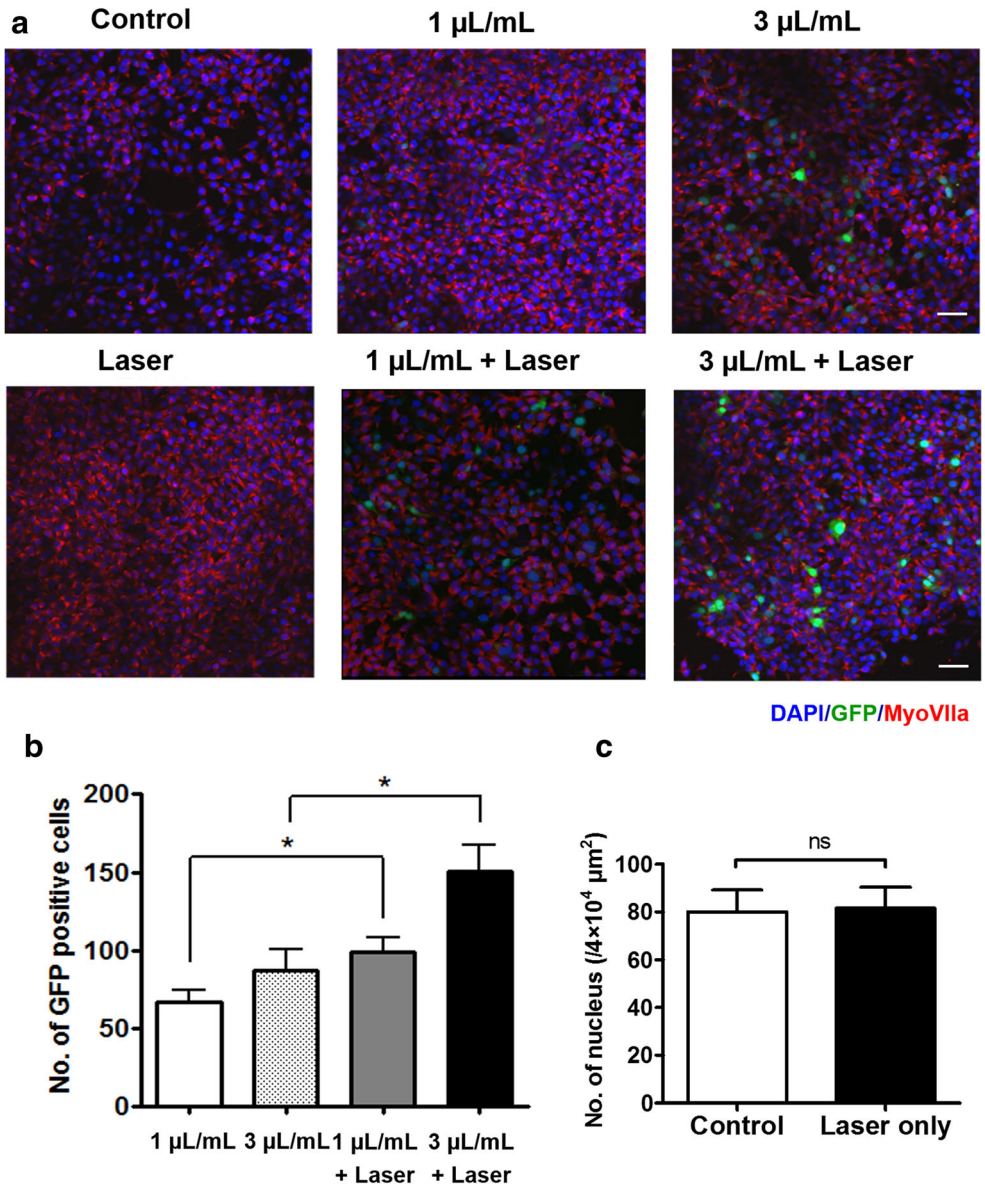

Fig. 3 Densities of GFP-positive cells. **a** Representative epifluorescence image used to analyze the density of GFP-positive cells. Auditory cell lines were stained with MyoVIIa (red), suggesting that these cells have unique biological characteristics of cochlea and vestibular hair cells. GFP-positive cells were not observed in the control or laser only groups. A higher concentration of Ad-GFP resulted in a higher density of GFP-positive cells. Laser-treated groups had a higher density of GFP-positive cells compared to the without laser groups (upper row) (scale bar 30 μm). **b** Quantitative analysis of GFP-positive cells 5 days after Ad-GFP inoculation revealed a significantly higher density of GFP-positive cells in the laser-treated groups ($N = 5–8$, $^*p < 0.05$)

viability of HEI-OC1 cell is not affected by laser exposure itself.

PBM increases the GFP expression using signal intensity analysis

Next, intracellular GFP expression was examined among GFP-positive cells. The intensity of GFP expression within the region of interest was determined using ImageJ software and revealed increased expression in the PBM groups relative to the non-laser groups (Fig. 4a). The GFP fluorescence intensities of the 1 μL/mL and 3 μL/mL virus-infected non-laser groups were 9.1 ± 2.7 and 13.4 ± 4.1, respectively. The GFP intensities of the 1 μL/mL virus + laser and 3 μL/mL virus + laser groups were 16.5 ± 5.2 and 22.1 ± 6.8, respectively. The significant increase in GFP fluorescence intensity in both groups was significantly increased after PBM (1 μL/mL virus + laser; observational power—0.779, β value—0.221, $p = 0.0159$; 3 μL/mL virus + laser; observational power—0.921, β value—0079. $p = 0.0012$) (Fig. 4b). This result suggests that PBM may improve gene expression in each cell after viral transfection.

RT-qPCR analysis

To further evaluate GFP expression, RT-qPCR analysis was conducted using a GFP antibody. The GFP gene was not expressed in the control or laser only groups without the virus. The amount of GFP expression in the laser-irradiated groups was statistically significantly increased by a factor of 1.2 approximately (1 μL/mL virus

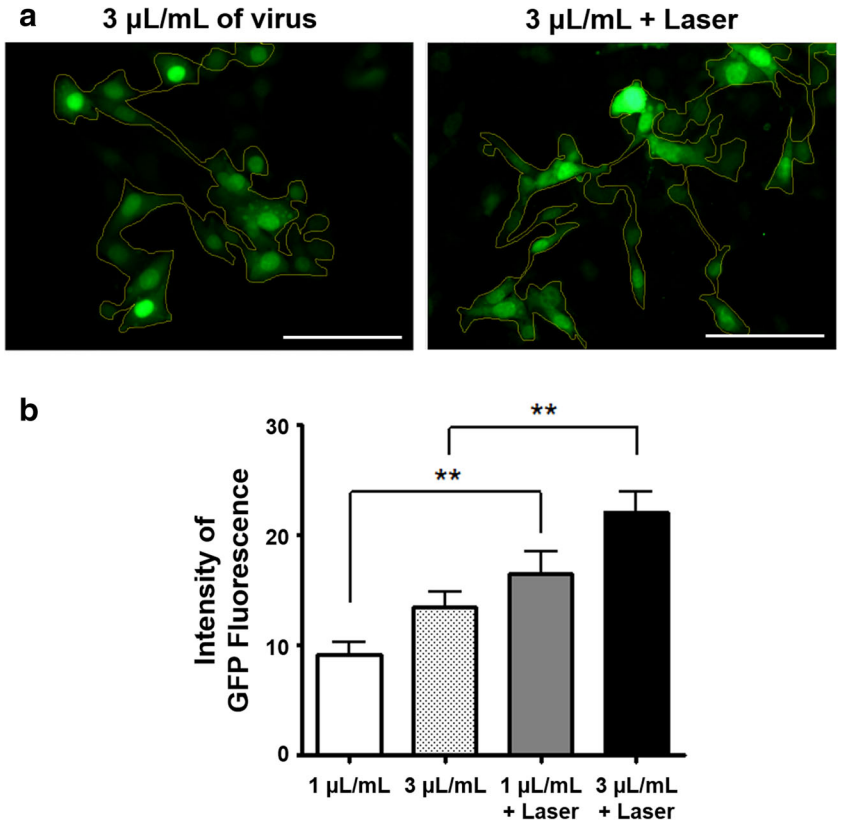

Fig. 4 Epifluorescence intensity of GFP-positive cells. **a** Representative image showing the measurement GFP expression (scale bar 100 μm). **b** The laser-treated group exhibited higher GFP expression compared to the group without laser application. A higher viral vector concentration was statistically associated with higher GFP expression. The laser-treated group exhibited significantly higher GFP expression compared to the without laser group ($N = 6–15$, $^{**}p < 0.01$). **c** HEI-OC1 cell density at 5 days after laser irradiation. Control and laser only groups showed no significant difference in HEI-OC1 cell density (mean ± SD; $n = 7–9$; ns = no significance)

+ laser; observational power—0.645, β value—0.355, p = 0.0159) (Fig. 5). These results provide additional evidence that gene expression following viral gene therapy is increased after PBM.

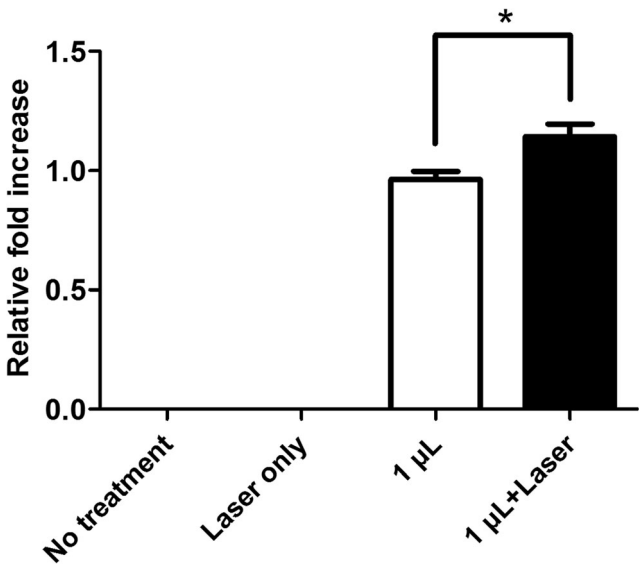

Fig. 5 RT-qPCR analysis of GFP. GFP was assessed using RT-qPCR. GFP was not detected in the groups without viral inoculation. The Ad-GFP with laser treatment group had an approximate 1.2-fold increase in GFP expression compared to the Ad-GFP without laser treatment ($N = 4–5$, $^{*}p < 0.05$)

Discussion

Summary and implications of the results

We verified that the Ad transduction process for HEI-OC1 cells does not alter target cell survival. We also found that the amount of GFP-expressing cells, which represents HEI-OC1 cells transfected with the Ad vector, and average GFP expression in each cell were increased after PBM. These results negate our null hypothesis and instead suggest that PBM enhances the efficiency of viral vector transduction and expression of the target protein, which is needed for the therapeutic effect of gene therapy.

Effects of laser application on viral vector transfection

Several studies have applied a laser to a virus. Therapeutic applications of laser phototherapy for herpes simplex virus (HSV) have been reported. Photodynamic therapy using various spectrums of laser, from visible light to near-infrared laser wavelengths, revealed a virucidal effect [29]. In the case of human immunodeficiency virus-1, low-level laser therapy using a 660-nm wavelength had an inhibitory effect on viral proliferation [30]. Additionally, murine cytomegalovirus was inactivated by ultrashort pulsed laser therapy [31]. These previous studies suggest that viral activation or proliferation is inhibited by laser application, and may be a potential method

of treating infections caused by the viruses mentioned above. Alternatively, our present study demonstrated an increased number of GFP-transfected cells using a viral vector and the possibility of increased gene expression of the target protein after PBM. Considering that HSV was the former virus that was used for gene delivery before the era of Ad and Ad-associated virus gene therapy [32], this surprising outcome could be related to differences of viral vector. In addition, laser parameters may contribute to these differences. A laser can be used to destroy pathological tissue with high-power energy [33, 34] and can be used to regenerate tissues in low- to medium-power energy [35, 36]. Alternatively, this difference could be due to the different characteristics of the target cells, as the hair cell of the inner ear (i.e., the cells that HEI-OC1 cells were modeled after) has very unique functions and properties.

PBM enhanced gene therapy for hearing loss (potential of clinical applications)

Sensorineural hearing loss is caused by damage to hair cells and the connecting peripheral nerves to the brain. These structures are highly vulnerable to certain injuries and once damaged they do not regenerate. Therefore, research in the regeneration of hair cells and functional recovery of the peripheral nerves involved in hearing is of paramount importance in the field of hearing. Gene therapy to regenerate or protect hair cells from external damage is a novel approach for hearing loss therapeutics. This study showed the feasibility of using PCM to enhance viral vector transduction for increased expression of protein. This technology, although promising, is still in its elementary stages and may not be directly translated to the clinic.

One consideration is a reliable technique for gene delivery to the organ of Corti, the inner ear structure composed of sensoriepithelium, containing hair cells, and non-sensoriepithelium. Since the inner ear is a fluid-filled cavity surrounded by a hard bone, access for viral vector transduction to the organ of Corti is challenging. Disruption of the surrounding structures may affect the homeostasis of inner ear fluid, resulting in damage to the cells necessary for sound conduction [37]. Further studies are needed to evaluate the use of laser enhanced viral vector transduction and modes of delivery in situ/in vivo using animal models that carry the biological and anatomical resemblance to the human cochlea.

In addition, selective delivery of light energy to the very small structures of the inner ear is admittedly very challenging, and a few animal studies have described the safety of the current modality [38]. Clinically, there are very few studies on the adverse effects of light energy on surrounding structures (e.g., facial nerve and labyrinth). Therefore, safety trials of the current laser technique should be performed to establish the laser gene therapy protocol in human subjects. To move this task from bench to clinic or even from cell to animal work, a very important factor must be considered: the delivery of laser energy to the organ of Corti, which is located in the cochlea and is situated inside the tympanic cavity. To reach the cochlea from the outside, sufficient energy during laser irradiation must pass the external ear and penetrate the tympanic membrane to be delivered to the target tissue. According to our previous study, the near-infrared laser, which has high penetration depth compared to other wavelengths, had a penetration rate of approximately 5–6% into the cochlea and has a proven effect of laser therapy that can restore damaged hair cells by drug- or noise-induced hearing loss [35, 36, 39]. The laser dosimetry used in this current study was calculated given these previous findings. The small laser power utilized for this study was designed for potential efficacy and safety in an animal model. Thus, positive outcomes in the present study using these laser parameters support possible benefit in studies in vivo.

Breakthroughs and limitations of PBM on gene therapy

The effects of PBM on gene transfection were observed, but the underlying mechanism is not clear. One possible mechanism could be the alteration of membrane integrity by PBM. Laser application can deliver external molecules such as exogenous DNA inside the target cell without manipulating the receptors and interactions of the cellular membrane [27]. A different study proposed that a pulsed laser beam could generate a submicrometer pore in the cellular membrane for this transient process [28]. Another explanation could be the direct effect of the laser to the virus. Laser phototherapy may enhance viral proliferation by increasing the synthesis of intracellular nucleic acids and ATP [16].

Although we demonstrated feasibility of using viral vector transduction with PBM enhancement, we did not observe the true therapeutic effects of gene therapy using the viral vectors with actual genes to repair the defected genes. To accomplish this, future in vivo studies using the gene to regenerate or protect hair cells in an animal model that resembles human disease conditions are necessary.

Conclusion

We demonstrated successful Ad-GFP viral transduction to auditory cells in vitro without toxicity. In addition, we showed the use of PBM to enhance GFP expression using low level laser treatment. Thus, the augmentation Ad viral transduction with PBM to increase protein expression has potential implications for an effective method of gene therapy for sensorineural hearing loss.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or live animals performed by any of the authors.

Informed consent This article does not contain any studies with human participants.

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