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ORIGINAL ARTICLE

Activation of Wnt/β-catenin signaling is involved in hair growth-promoting effect of 655-nm red light and LED in in vitro culture model

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Abstract

Activation of the Wnt/β-catenin signaling pathway plays an important role in hair follicle morphogenesis and hair growth. Recently, low-level laser therapy (LLLT) was evaluated for stimulating hair growth in numerous clinical studies, in which 655-nm red light was found to be most effective and practical for stimulating hair growth. We evaluated whether 655-nm red light + light-emitting diode (LED) could promote human hair growth by activating Wnt/β-catenin signaling. An in vitro culture of human hair follicles (HFs) was irradiated with different intensities of 655-nm red light + LED, 21 h7 (an inhibitor of β-catenin), or both. Immunofluorescence staining was performed to assess the expression of β-catenin, GSK3β, p-GSK3β, and Lef1 in the Wnt/β-catenin signaling. The 655-nm red light + LED not only enhanced hair shaft elongation, but also reduced catagen transition in human hair follicle organ culture, with the greatest effectiveness observed at 5 min (0.839 J/cm^2). Additionally, 655-nm red light + LED enhanced the expression of β-catenin, p-GSK3β, and Lef1, signaling molecules of the Wnt/β-catenin pathway, in the hair matrix. Activation of Wnt/β-catenin signaling is involved in hair growth-promoting effect of 655-nm red light and LED in vitro and therefore may serve as an alternative therapeutic option for alopecia.

Keywords 655 nm · Low-level laser therapy · Hair · Wnt/β-catenin signaling

Abbreviations

LLLT	Low-level laser therapy
HF	Hair follicle
LED	Light-emitting diode
MK	Matrix keratinocyte
GSK3β	Glycogen synthase kinase-3β
p-GSK3β	Phospho-GSK3β
Lef1	Lymphoid enhancer factor 1
DPC	Dermal papilla cell
ROS	Reactive oxygen species
TCF/LEF	T cell factor/lymphoid enhancer factor
AGA	Androgenetic alopecia

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Introduction

Because the different types of alopecia involve physical and mental disorders affecting more than half of the global population, more efficacious treatment options are required. Recently, low-level laser therapy (LLLT) was demonstrated to stimulate hair growth in individuals with male-pattern hair loss, female-pattern hair loss, and alopecia areata with potentially better outcomes and minimal side effects [1–3]. The FDA has stated that LLLT devices operating at a wavelength of 655 nm are both safe and efficacious in patients who do not respond, or are not tolerant to conventional treatment methods [4, 5]. In addition, light-emitting diodes (LEDs) are safe and portable, emit quasi-monochromatic light with low coherence, and use non-thermal energy [6]. Although LLLT is now widely used for the treatment of a variety of diseases, its use as a therapeutic modality still remains controversial for two reasons: first, the precise biochemical mechanisms are poorly

known. Second, the optimal optical parameters, such as wavelength, irradiance, fluence (energy density), and power density, of laser therapy are different, and further experimentation is required for therapeutic applications [7].

The hair follicle (HF) is a highly sensitive "mini-organ," which is composed of epithelial and mesenchymal tissues, and undergoes cyclic transformation, including rapid growth (anagen), regression (catagen), and resting (telogen) [8]. Even after their removal from the human scalp, HFs still maintain most of their crucial in vivo characteristics in hair follicle organ culture. For example, HFs continue to produce keratinized hair fibers, sustain the cyclical transformation, and maintain protein synthesis [9]. Thus, it is important to evaluate the effects of LLLT on cultured human HFs without interference.

The Wnt/β-catenin signaling pathway plays the most important role in the initiation of the growth, and development of hair follicles [10, 11]. Recent studies involving knockout mouse models have also revealed that the Wnt/β-catenin-mediated signaling pathway is critical to the hair growth cycle [12, 13]. In addition, a previous study reported that the Wnt10b protein was specifically expressed in the matrix cells of HFs in the anagen stage, and stimulated HF regrowth by activating the Wnt/β-catenin signaling pathway [14]. However, whether Wnt/β-catenin signaling is involved in the underlying mechanism of the effect of LLLT on hair growth has not been explored thoroughly.

Unlike other laser therapies, LLLT acts by inducing a photochemical reaction in cells without producing any thermal effect, or causing irreversible damage [7]. Effects, such as proliferation, migration, oxygenation, and adhesion, which are induced by LLLT, may allow for hair regrowth, and the subsequent reversal of the growth of hair follicles from the dormant telogen stage to the active anagen stage [15–17]. In this study, we investigated whether red light at 655 nm together with an LED affected the growth of in vitro cultured human HFs, which were exposed to different doses of light; we also explored the underlying mechanism by analyzing the key proteins in the Wnt/β-catenin signaling pathway. The results demonstrated that LLLT promoted the growth of human HFs in vitro with the highest efficiency observed at 0.839 J/cm^2, possibly by activating the Wnt/β-catenin signaling pathway partly.

Materials and methods

Laser treatment

The SPARK hair growth system uses 21 laser diodes (655 nm) and 26 LEDs (655 nm) as LLLT sources. The wavelength was remeasured with a QE65000 spectrograph (Ocean Optics, Duiven, The Netherlands) and was confirmed to show a peak wavelength of 655 nm. The operating currents were the same and peak power was 5 mW for the laser diodes and LEDs. The total effective irradiated area was approximately 84 cm^2. Therefore, the energy density was respectively set to 0.839 J/cm^2 (exposure time 5 min), 1.679 J/cm^2 (exposure time 10 min), and 2.518 J/cm^2 (exposure time 15 min).

Human hair follicles organ dissection and culture

Hair follicle samples were provided by 10 healthy volunteers (average age, 34 ± 9.0 years) from discarded scalps of the occipital region without infection, alopecia, or other scalp diseases obtained following neurosurgery. The samples were rinsed with saline, D-Hanks buffer containing 2% antibiotics (streptomycin and penicillin), and 1% antibiotics in sequence. The samples were carefully dissected into single HFs under a dissecting microscope (Leica, Wetzlar, Germany) and only isolated anagen VI follicles were used in this study [18].

As previously described [19], isolated human scalp hair follicles maintained in free-floating cultures were cultured in Williams E medium (Gibco BRL, Grand Island, NY, USA) containing 10 mg/L insulin, 10 μg/L hydrocortisone, 2 mM glutamine, 2 mM HEPES, 100 U/mL penicillin, and 100 mg/L streptomycin in individual wells of 24-well plates at 37.8 °C in a 5% CO_2 atmosphere. Hair follicles that grew to lengths of 0.3–0.5 mm after 24 h were selected [18] and divided into 4 groups for different LLLT exposure times: 5 min (0.839 J/cm^2), 10 min (0.839 J/cm^2), 15 min (2.518 J/cm^2) group, and control group (0 min). Each group contained 18–20 HFs. Laser treatment was performed on alternate days and elongation of the hair shaft was measured. An inverted microscope (NIKON, Tokyo, Japan) was used to acquire images of HFs every second day. After 5 days, HFs from each group were collected and fixed in 4% paraformaldehyde.

Immunofluorescence staining

Immunofluorescence staining was performed to evaluate the proliferation of hair matrix keratinocytes (MK) and assess the molecular expression of β-catenin, glycogen synthase kinase-3β (GSK3β), phospho-GSK3β (p-GSK3β), and lymphoid enhancer factor 1 (Lef1), i.e., molecules of the Wnt/β-catenin signaling pathway, from each cultured hair follicle. Immunoreactivity for Ki67 was used as an indicator of cell proliferation and all antibodies (Table 1) including those for ki67, β-catenin, GSK3β, p-GSK3β, and Lef1 were obtained from Abcam (Cambridge, UK). The collected HF samples were embedded in paraffin and sectioned. Then, the sections were blocked with bovine serum albumin (Jetway, China) and incubated with antibodies (Abcam) overnight at 4 °C. Next, the sections were incubated with Alexa Fluor 594 (goat anti-mouse; Invitrogen, USA) or Alexa Fluor 488 (goat anti-rabbit; Invitrogen) secondary antibodies for 1 h at room temperature

Table 1 Information of antibodies used in the Immunofluorescence staining

	Category	Concentration	Secondary antibodies
Ki67	Mouse monoclonal	1:100	Alexa Fluor 488
β-catenin	Rabbit monoclonal	1:500	Alexa Fluor 594
GSK3β	Rabbit monoclonal	1:500	Alexa Fluor 594
p-GSK3β	Rabbit monoclonal	1:100	Alexa Fluor 594
Lef1	Rabbit monoclonal	1:500	Alexa Fluor 594

(20 °C). Counterstaining was performed with 4′,6-diamidino-2-phenylindole (DAPI; Beyotime Biotechnology, China). We used a fluorescence microscope (NIKON ECLIPSE TI-SR) to acquire images. The fluorescence intensities of the molecules listed above in the hair matrix were converted into gray values and measured using Image J software (NIH, Bethesda, MD, USA).

Statistical analysis

Groups of data were expressed by the means ± standard error of the mean (SEM) and the results were analyzed by one-way analysis of variance. Least significant difference analysis was performed to compare groups. A p value was < 0.05 indicated a significant difference.

Results

655-nm red light + LED promotes human hair growth in in vitro culture model

According to the results, the growth rate in 5 min and 10 min group was approximately linear until the end of the experiment. Compared to average hair shaft growth, HFs treated for 5 and 10 min were much longer than HFs without laser irradiation ($^{**}p < 0.01$), while the length of HFs treated for 15 min did not differ from the control group ($p > 0.05$); HFs irradiated for 5 min grew faster than those in the 10 min-irradiated group ($^{*}p < 0.05$) (Fig. 1a, b).

In early catagen, HFs show a remarkably thin hair matrix, oval dermal papilla, and low melanin levels [19]. In our experiment, these changes occurred in HFs in the control group on the 7th day, while HFs in the 5, 10 and 15 min-irradiated groups appeared anagen till 11 days (Fig. 1c). This indicates that the catagen transition of HFs was postponed by laser treatment.

The expression level of Ki67 in hair matrix area is important for assessing the proliferation of hair follicle. Immunofluorescence staining was used to confirm proliferation enhancement by laser treatment (Fig. 1d). HFs were collected after 5 days of treatment and the proportion of Ki67+ cells (red fluorescence) relative to DAPI-stained cells (blue fluorescence) was used to verify this effect. Compared to 24.7% Ki67+ cells of the hair matrix without irradiation, the 5 and 10 min-treated hair matrix contained 62.5 and 42.3% of Ki67+ cells, respectively (Fig. 1e). Therefore, proliferation was significantly increased by laser treatment ($^{**}p < 0.01$) and the 5 min-treated group showed a greater effect.

To evaluate the molecular expression of the Wnt/β-catenin signaling pathway in laser-treated hair matrix treated, immunofluorescence staining was conducted to examine the expression quantity of important signaling molecules such as β-catenin, GSK3β, p-GSK3β, and Lef1. According to our results, β-catenin (green fluorescence) was expressed both in the cytoplasm and nucleus, and GSK3β, p-GSK3β (green fluorescence) were mainly expressed in the cytoplasm, while Lef1 (green fluorescence) was in the nucleus (Fig. 1f). The immunofluorescence intensities (mean optical densities) of β-catenin, p-GSK3β, and Lef1 in the hair matrix area of 5 and 10 min-treated HFs were significantly higher than those in the control group and 15 min-treated group. In addition, the intensities of GSK3β showed no significant differences among groups (Fig. 1g).

655-nm red light + LED promotes human hair growth in in vitro culture model by activating Wnt/β-catenin signaling

To further confirm the effect of 655-nm red light + LED on Wnt/β-catenin pathway activation, 5 min-irradiated HFs were treated with the β-catenin inhibitor 21H7, which has been demonstrated to potently reduce the stability, suppressing nuclear translocation and transcriptional activity of β-catenin [20]. Our initial findings showed that 21H7 functioned effectively at a concentration of at least 4 μM. Additionally, 4 μM 21H7 inhibited the growth of hair follicles (Fig. 2a, b), accelerated the catagen transition (Fig. 2c), and decreased the proliferation of HF matrix keratinocytes (Fig. 2d, e); however, HFs cultured with 4 μM 21H7 and then irradiated with 5 min of laser showed no significant difference from the control group and showed a significant difference with 21H7 group and 5 min-irradiated group for these parameters (2a-2e). The growth rate of HFs in 21H7 + 5 min group was significantly slower than 5 min-irradiated group and faster than 21H7 group (all $^{*}p < 0.05$). Finally, the expression of β-catenin, p-GSK3β, and Lef1 in the hair matrix was suppressed by 21H7 compared to in the control group, while the 21H7 +

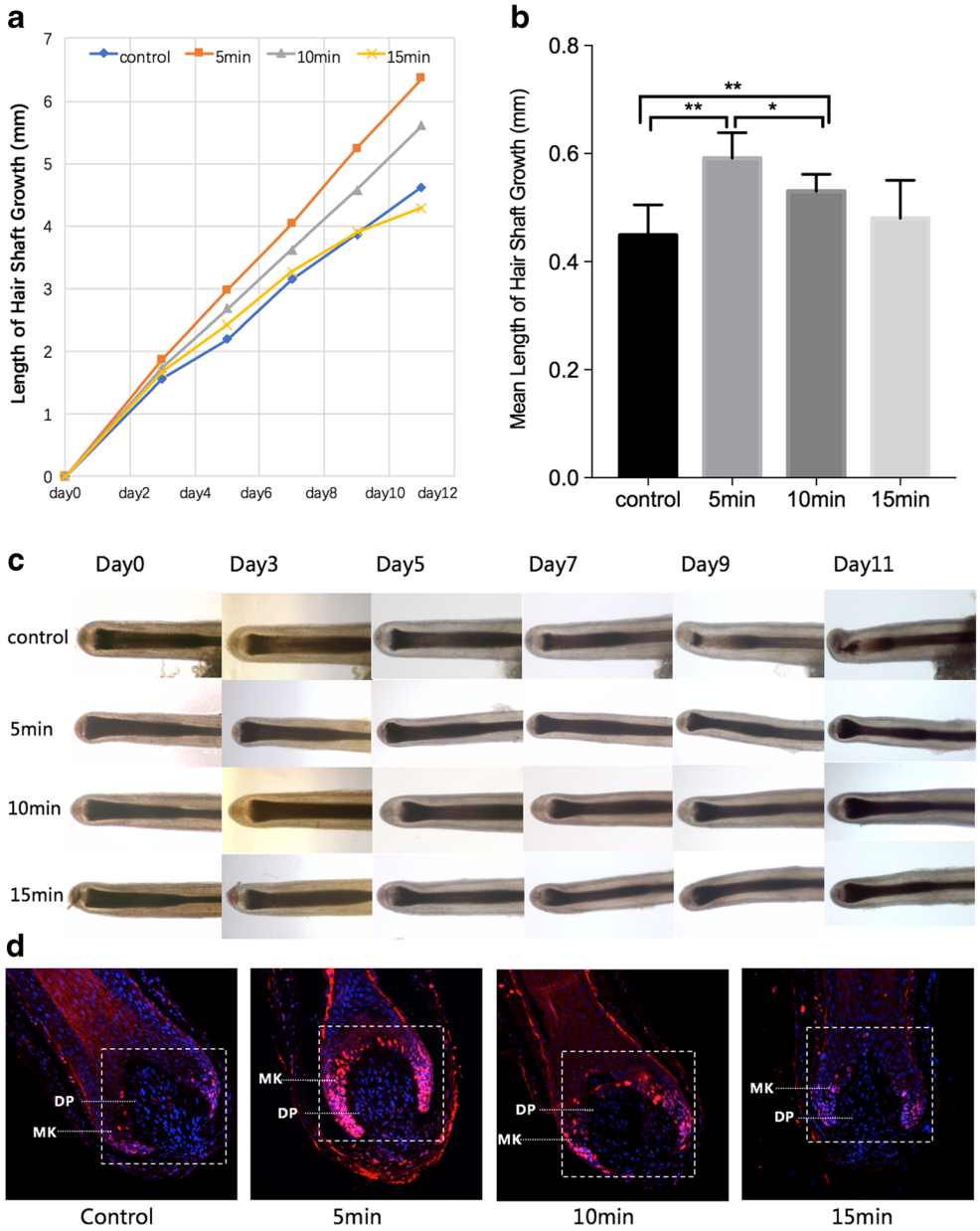

Fig. 1 Hair shaft elongation enhancement and catagen transition postponement by 655-nm red light + LED in in vitro culture of human HFs. **a**, **b** Exposure to 655-nm red light + LED for 5 and 10 min enhanced hair shaft elongation and **c** reduced catagen transition compared to in the control, with the greatest effectiveness at 5 min. **d** The proliferation (Ki67, red fluorescence) of matrix keratinocytes (MK) was enhanced by 655-red light + LED. **e** Ki67 + cells were counted and normalized to DAPI-stained cells. **f** 655-nm red light + LED irradiated for 5 and 10 min enhanced expression of β-catenin, p-GSK3β, and Lef1 in hair matrix (green fluorescence). **g** For quantitative analysis, the intensities of β-catenin, GSK3β, p-GSK3β, and Lef1. The results are shown as the means ± SEM, and $*p < 0.05$ and $**p < 0.01$ compared with the control

5 min group did not show this effect (2f, 2g). More importantly, expressions of key markers in the Wnt/β-catenin signaling pathway in 21H7 + 5 min group are significantly lower than 5 min-treated laser group (all $*p < 0.05$).

Discussion

Herein, we systematically performed an in vitro hair follicle organ culture with red light at 655 nm in combination with LED treatment, and investigated the corresponding underlying mechanisms and optimal parameters preliminarily. The results of this study revealed a considerable increase in hair growth following the treatment with red light at 655 nm together with LED, which involved the activation of the Wnt/β-catenin signaling pathway in vitro, with the highest efficiency observed at 5 min (0.839 J/cm^2).

The accelerated progression of the hair growth cycle prompted us to study how hair follicles sense red light at 655 nm in combination with LED. Isolated follicle culture is

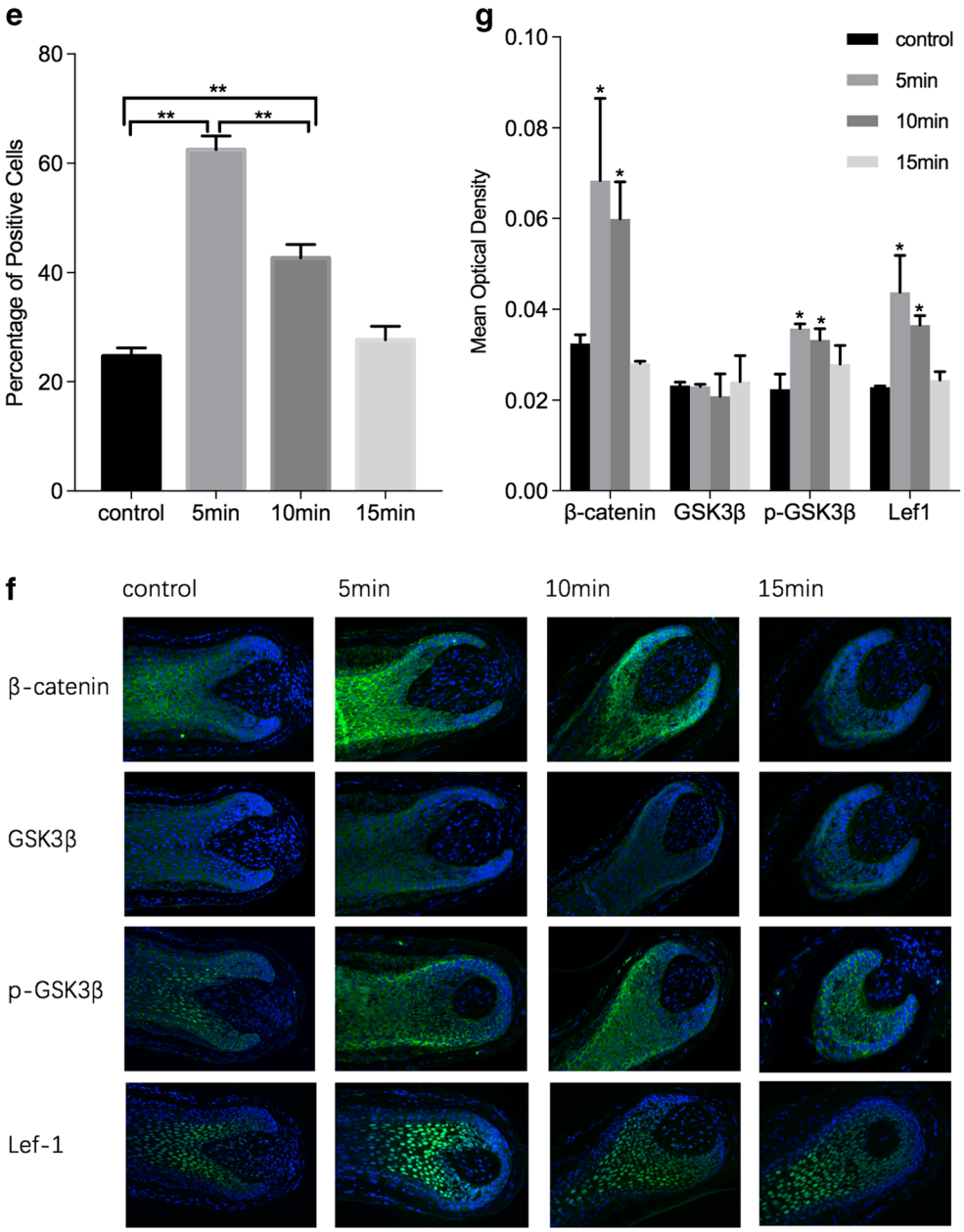

Fig. 1 (continued)

a good model for retaining the functional and anatomical relationships between the dermal papilla and hair matrix, epithelial, and mesenchymal components to explore hair growth [21]. During ex vivo organ culture of hair follicles, the hair follicles enter catagen phases over 8–10 days and their growth rate would slow down [19]. In this study, we used human samples, and experimentally demonstrated that the growth of human HFs was promoted by red light at 655 nm together with LED. The results are similar to those of previous studies that were conducted using LLLT with light at 635 and 660 nm and their combination in vitro [5, 22]. Additionally, this is the first study to demonstrate that the rate of MK proliferation was increased by LLLT, as shown by Ki67 immunostaining, and catagen transition was reduced in the in vitro culture model. In normal hair follicles in the anagen stage, proliferating cells are observed only in the hair matrix [23]. The duration of anagen determines hair length, and the proliferation and differentiation of MKs at the base of follicles [24]. Once the reservoir of MKs decreases, the hair shaft and inner root sheath differentiate slowly, and the follicle enters a regressive phase known as catagen [25]. We carefully concluded that LLLT enhanced the proliferation of MKs, prolonged the duration of the anagen phase, and induced the rapid elongation of hair shafts.

Following these findings, we further investigated the mechanism of LLLT. The Wnt/β-catenin signaling pathway is not only the largest contributor to HF morphogenesis and hair

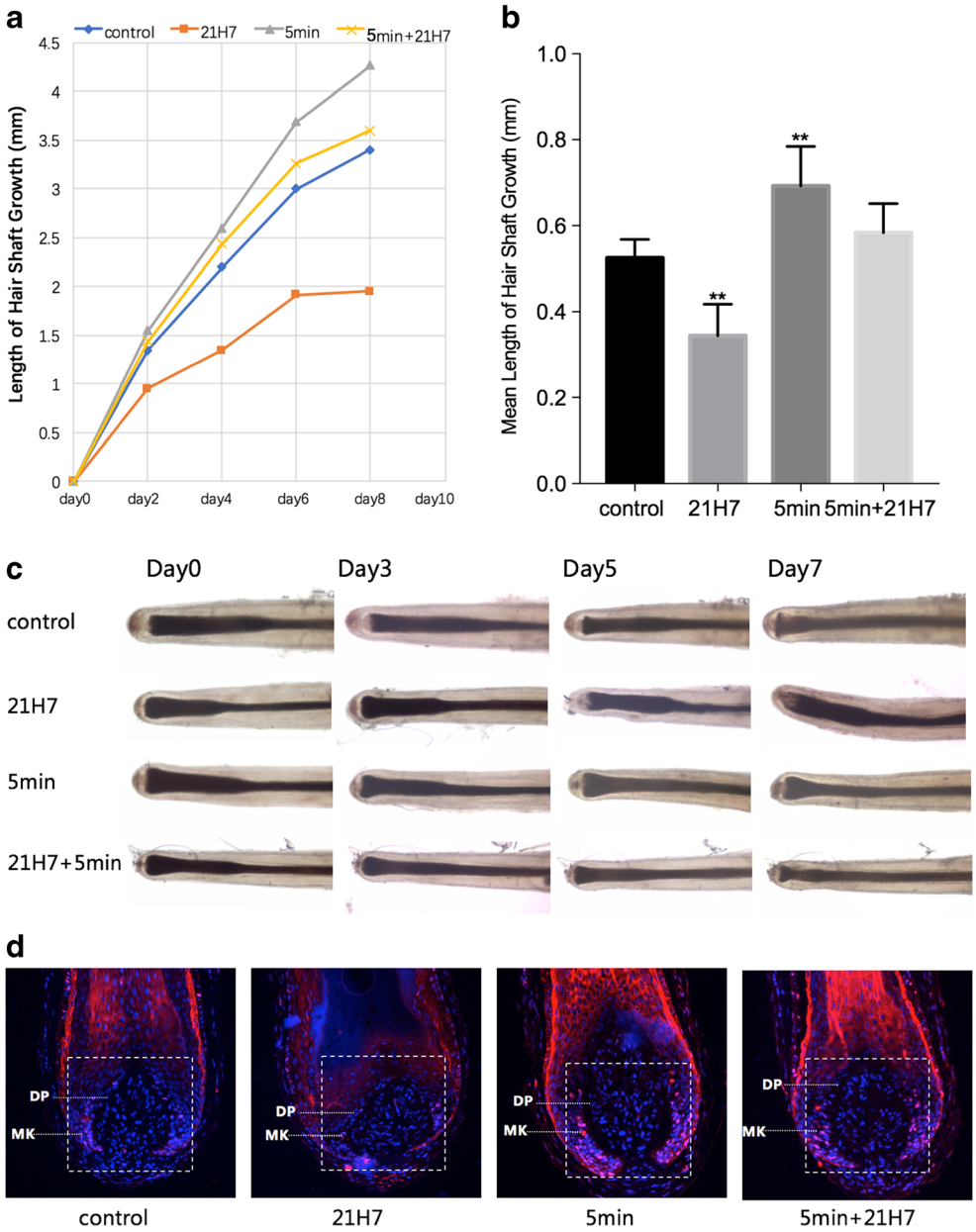

Fig. 2 655 nm red light + LED partly eliminated the HF growth-alleviating effect of 21H7 probably by activating Wnt/β-catenin signaling (**a**–**g**). The results are shown as the means ± SEM, $*p < 0.05$ and $**p < 0.01$ compared with the control

growth [26–28], but also crucially important to the hair growth cycle; the regeneration of the hair cycle and the maintenance of the anagen phase are β-catenin-dependent [10, 29]. It also reported that the Wnt/β-catenin signaling pathway is critical to hair loss of different types, such as androgenetic alopecia (AGA) [13, 30]. When the pathway is activated, Wnt binds to receptors in the cell membrane, and GSK3β is phosphorylated, and β-catenin accumulates in the cytoplasm and then translocated into the nucleus, leading to increased Lef1 expression [31, 32]. Our results showed that red light at 655 nm together with LED enhanced the expression of β-catenin, p-GSK3β, and Lef1, which are involved in the Wnt/β-catenin signaling pathway in the hair matrix. Consistent with our results, Tiran Zhang et al. conducted a study on C3H/HeJ mice, and demonstrated higher expression levels of Lef1 and β-catenin in the LLLT group, which indicated a crucial role of LLLT in the Wnt/β-catenin signaling pathway [33]. In addition, we discovered that HFs cultured with 21H7 + laser (5 min) showed a significant difference with 21H7 group and the 5 min-treated laser group. Similarly, the expression of β-catenin, p-GSK3β, and Lef1 in the Wnt/β-catenin signaling pathway in 21H7 + 5 min group is significantly lower than 5 min-treated laser group. It indicates the HF growth-suppressing and β-catenin-inhibiting

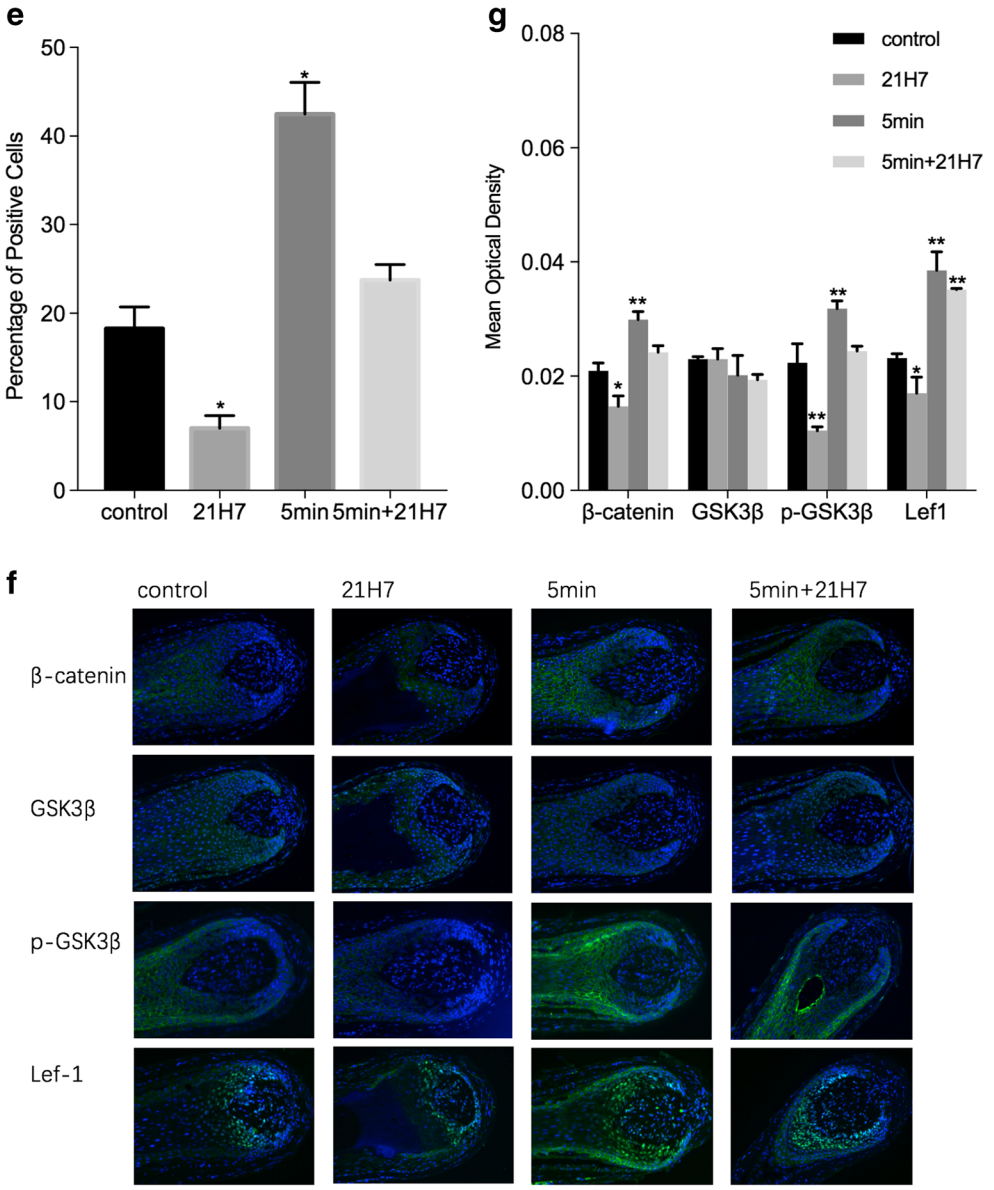

Fig. 2 (continued)

effects of 21H7 are partly eliminated by the treatment with red light at 655 nm together with LED, which suggests that the Wnt/β-catenin signaling pathway could probably be activated by LLLT in the hair matrix and it might not be the only signaling way in the process of hair growth.

Activation of the Wnt/β-catenin result in the proliferation of hair MKs, which explains why LLLT enhanced the proliferation of MKs [34]. After irradiation, the hair matrix cells were the first to receive the signal, and rapidly undergo proliferation [27, 35]. Yi-Shuan Sheen et al. reported that red light could increase the expression of β-catenin and Lef1 in dermal papilla cells (DPCs), leading to outer root sheath keratinocytes proliferation and acceleration of anagen entry; it also suggested enhanced Wnt signaling in DPCs [36]. The dermal papilla, which is derived from the mesenchyme, is an inductive structure that sends and receives signals based on the continuous interaction with the hair matrix epithelium [37]. The stimulatory signals that are produced by the dermal papillae are received by the hair matrix epithelium, and then, proliferative cells (matrix) at the base of follicles continue to divide, producing progeny cells that terminally differentiate to form growing hair [23, 38]. Most importantly, we can speculate that LLLT directly stimulates the proliferation of hair MKs by enhancing the activation of certain pathways including the Wnt/β-catenin signaling pathway in the hair matrix, and indirectly through interactions of hair matrix and dermal papillae. However, further studies are required to support this speculation.

In summary, we discovered that red light at 655 nm together with LED promoted hair growth from human HFs in vitro by partly activating the Wnt/β-catenin signal pathway probably, which indicate the important role of this treatment technique for alopecia, and provides a foundation for further studies on the mechanisms of LLLT. Furthermore, the evidence is exclusively correlative now and it is necessary to explore the exact relationship between LLLT and the Wnt/β-catenin signaling pathway in vitro in the subsequent investigation.

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Compliance with ethical standards

Study protocols were approved by the Institutional Research Ethics Committee, Sun Yat-sen University (No. [2015]2-174), and written informed consent was obtained from all subjects. All experimental procedures using human tissues were performed according to the principles described in the Declaration of Helsinki.

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

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