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Hair Growth Promoting Effects of 650 nm Red Light Stimulation on Human Hair Follicles and Study of Its Mechanisms via RNA Sequencing Transcriptome Analysis

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Background: Androgenetic alopecia (AGA) leads to thinning of scalp hair and affects 60%~70% of the adult population worldwide. Developing more effective treatments and studying its mechanism are of great significance. Previous clinical studies have revealed that hair growth is stimulated by 650-nm red light.
Objective: This study aimed to explore the effect and mechanism of 650-nm red light on the treatment of AGA by using *ex vivo* hair follicle culture.
Methods: Human hair follicles were obtained from hair transplant patients with AGA. Hair follicles were cultured in Williams E medium and treated with or without 650-nm red light. Real-time RT-PCR and immunofluorescence staining were used to detect the expression level of genes and proteins in hair follicles, respectively. RNA-sequencing analysis was carried out to reveal the distinct gene signatures upon 650 nm treatment.
Results: Low-level 650 nm red light promoted the proliferation of human hair follicles in the experimental cultured-tissue model. Consistently, 650 nm red light significantly delayed the transition of hair cycle from anagen to catagen *in vitro*. RNA-seq analysis and gene clustering for the differentially expressed genes suggests that leukocyte transendothelial migration, metabolism, adherens junction and other biological process maybe involved in stimulation of hair follicles by 650-nm red light treatment.
Conclusion: The effect of 650-nm red light on *ex vivo* hair follicles and the transcriptome set which implicates the role of red light in promoting hair growth and reversing of miniaturization process of AGA were identified.

Keywords: 650-nm red light, Androgenetic alopecia, Hair follicle, Low-level laser therapy, RNA sequencing

INTRODUCTION

Androgenetic alopecia (AGA) is a common condition that affects approximately 80% of Caucasian males and 50% of females respectively, by the age of 70 years and has also been associated with hair loss[1,2]. AGA is characterized by gradual transformation of terminal hairs to vellus-like hairs and results in thinning hairs[3,4]. The structure of a hair contains two parts: the hair shaft present above the epidermis and the hair follicle (HF) present below the epidermis. Besides, HFs are complex mini-organs undergoing cycles of anagen (growth), catagen (degeneration) and telogen (rest)[5]. A variety of genes and pathways are involved in the pathogenesis of AGA, including Wnt/β-catenin, immune and inflammatory responses, Janus-

activated kinase, sonic hedgehog, etc[5-7]. Among them, Wnt/β catenin signalling pathway, which is specially important for the hair cycle and hair growth, has been shown to be negatively influenced by the increased androgen in AGA patients[8]. Some inflammatory markers were also found to be specifically expressed in AGA lesional bald scalp compared to the non-lesional scalp[9]. These differences in gene expression and signalling pathways ultimately led to the pathophysiology of AGA, such as aberrant follicle cycle, follicle miniaturization, and loss of hair. Therefore, controlling the hair cycle and promoting hair growth through maintaining anagen and shortening catagen or telogen is critical in the treatment of thinning hairs or hair loss in AGA.

Hair loss can have significant effects on patients' psychological health and quality of life. Hair transplantation and growth factor injection can be used to treat hair loss, but so far, only two drugs (minoxidil and finasteride) have been approved by the Food and Drug Administration (FDA) for the treatment of hair loss. However, these drugs have demonstrated temporary effects or their use for women has been restricted. Therefore, there is an increasing demand for novel treatments that prevent the progression of hair loss, facilitate hair regrowth, and have minimal side effects.

It has been reported that specific wavelengths of laser light have biological effects, and near-infrared light has been used to stimulate stem cell proliferation and differentiation[10]. Recently, low-level laser (or light) therapy (LLLT) has been introduced as a therapeutic option for people who fail to, or are unwilling to use traditional medical therapy or undergo surgical treatment for hair loss[11]. LLLT appears to be a safe and effective treatment in AGA[12,13] and LLLT devices have become commercially available to AGA patients. Strong evidence suggests that LLLT stimulates anagen re-entry of telogen HFs and prolongs the duration of the anagen phase[14,15]. Additionally, LLLT acts on mitochondria to increase reactive oxygen species levels, adenosine triphosphate (ATP) production, and induction of transcription factors that activate genes and produce proteins which are useful to the multiplying cell[16-18].

Previous studies have indicated that 650-nm red light is the most effective and practical way for stimulating hair growth via LLLT treatment. However, mechanisms by which LLLT reduces hair loss are not yet clearly understood. Therefore, we examined the effects of 650 nm light on hair growth using cultured human HFs and identified potential transcriptional mechanisms via RNA sequencing (RNA-seq) analysis.

MATERIALS AND METHODS

Human hair follicle organ dissection and culture

Occipital scalp HF units were obtained during the hair transplant of AGA patients. All participants signed informed consents and were notified about the study before participation. The study was approved by ethics committee of Huashan Hospital of Fudan University (ethical approval number 2019M-008) and was performed in accordance with the principles embodied in the declaration of Helsinki. The informed consent was obtained from all patients who provided samples. Anagen VI follicles were isolated from the human scalp and cultured in William's E medium (Gibco BRL, Grand Island, NY, USA) containing 2 mM glutamine, 10 ng/ml hydrocortisone, 100 U/ml penicillin, and 100 mg/ml streptomycin in individual wells of 12-well plates at 37.0°C in a 5% CO_2 incubator. HFs that grew to lengths of 0.3~0.5 mm after 24 hours were selected for subsequent LLLT treatment experiments[19].

Hair follicle treatment with 650 nm light

Light emitting diodes (LEDs; 650 nm) (CHARMWIN, Beijing, China) were used as red light sources. The wavelength was re-measured with a Spectral Flickering Irradiance Meter (SFIM-300_V310, Hangzhou, China) and was confirmed to show a peak wavelength of 650 nm. The energy density was 0.8 J/cm^2 (exposure time: 5 minutes) and 1.6 J/cm^2 (exposure time: 10 minutes). The selected HFs were divided into 3 groups, with different LLLT exposure times: 5 minutes, 10 minutes, and control (0 minute). Each group contained 6 HFs and laser treatment occurred on alternate days. Mediums were changed every other days and images of HFs were also captured every alternate day using a stereomicroscope (Olympus, Tokyo, Japan), to measure the elongation of hair shaft. Twenty-four hours after the first 650-nm LED treatment, HFs from each group were collected and fixed in 4% paraformaldehyde for immunofluorescence staining or lysed with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for RNA extraction.

RNA isolation, reverse transcription and real-time PCR

Total RNA was extracted from HFs using TRIzol according to the manufacturers' instruction (Invitrogen). Reverse transcription was performed using a High Capacity cDNA Reverse

Transcription Kit (Applied Biosystems, Waltham, MA, USA) according to the manufacturers' protocol. SYBR Premix Ex Taq (TakaRa Biotech, Tokyo, Japan) and an ABI Prism 7900 Detector System (Applied Biosystems) were used to perform real-time RT-PCR. The expression levels of the housekeeping gene *GADPH* were assessed as an internal control. The data obtained from the assays were analyzed using SDS 2.3 software (Applied Biosystems).

Immunofluorescence staining

HF samples were embedded in paraffin and sectioned. The sections were blocked with bovine serum albumin and incubated with an antibody to Ki67 (Abcam, Cambridge, UK), an indicator of cell proliferation, overnight at 4°C. The sections were then incubated with Alexa Fluor 488 (Invitrogen) secondary antibody for 1 h at room temperature, and cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Beyotime Biotechnology, Nanjing, China). Stained sections were viewed and photographed using fluorescence microscopy (Olympus).

RNA-sequencing data analysis

Up to 1~2 μg of total RNA was extracted from untreated or 650 nm light-treated HFs and used to prepare sequencing libraries with a TruSeq RNA Library Prep Kit (Illumina, San Diego, CA, USA). A HiSeq X Ten genome analyzer (Illumina) was used to perform the RNA sequencing. FastQC was used to assess sequence quality and FASTX-Toolkit was used to filter out the low-quality reads. The data was analyzed by Kallisto (version 0.44.0; Invitrogen) for quick transcript quantification without alignment. The index we used was downloaded from the Ensembl database (Homo_sapiens.GRCh38.cdna.all.fa.gz) and was built in the *Kallisto index* (a Kallisto module). We ran *Kallisto quant* with a bootstrap parameter-b 100 to produce an h5 file as input for sleuth analysis. The expression level was quantified by transcript per million (TPM) and counts. Sleuth (version 0.30.0) and DEseq2 (version 1.20.0) analysis were utilized to detect the differentially expressed transcripts (DETs) or genes (DEGs). A padj value <0.1 (adjusted by false discovery rate) was set to indicate DETs and the genes encoded by these transcripts were considered DEGs. The gene ontology (GO) enrichment analysis and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of DEGs were performed using the R package cluster Profiler (version 3.8.1; R Foundation for Statistical Computing, Vienna, Austria). The R package ggplot2 (version 3.0.0) was used to draw the bar plot and the volcano plot, while the pheatmap (version 1.0.12) was used to draw the heatmap.

Statistical analysis

All statistical analyses of data from our experiments were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). The independent two-group t-test and one-way ANOVA test were used for the evaluation of significance between different groups, and a *p*-value of less than 0.05 was considered significant. All experiments were performed in triplicate.

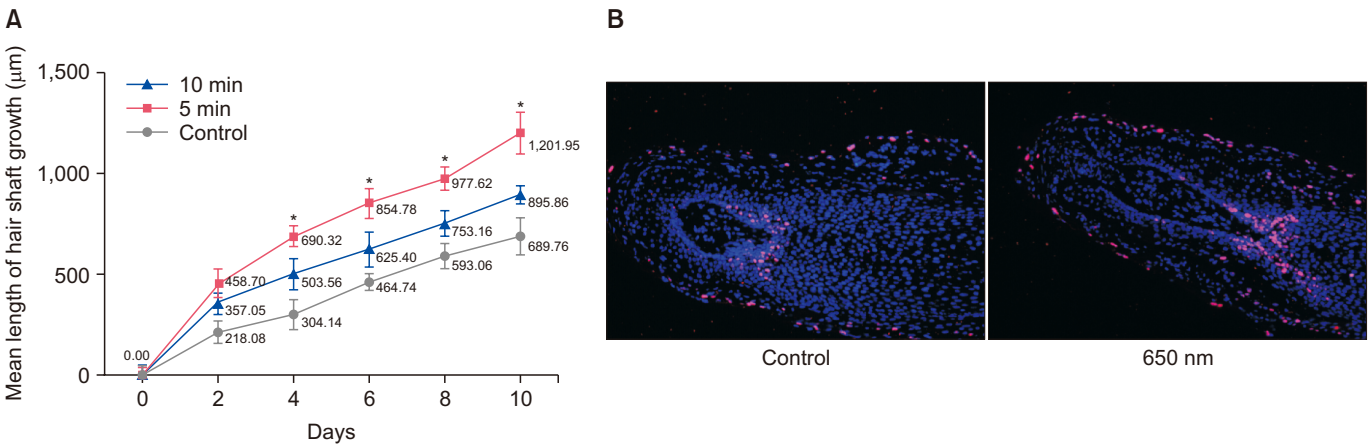

Fig. 1. Hair shaft elongation and cell proliferation detected in cultured human hair follicles. (A) Hair shaft length was measured every other day. (B) immunofluorescence staining images showing the localization of the cell proliferation marker Ki67, and the cell nucleus was stained with DAPI.

RESULTS

Treatment with 650-nm red light promoted human hair growth in an *ex vivo* culture model

The isolated human scalp HFs were cultured *in vitro* and divided into three groups (control, 5 minutes, 10 minutes) and the elongation of hair shaft was measured every other day. Compared to the control group, both 5 minutes and 10 minutes treatment with 650 nm red light increased the mean length of hair shaft growth (Fig. 1A). Moreover, HFs treated for 5 minutes showed significantly increased length compared to the control group ($p<0.05$), while there was no significant difference between the length of HFs treated for 10 minutes and the control group ($p>0.05$). Ki67 is a typical marker of cell proliferation and the expression level of Ki67 in the hair matrix area was used to assess the proliferation of HFs. As shown in Fig. 1B, proliferation enhancement by treatment with 650-nm red light was confirmed via immunofluorescence staining. HFs were collected after 4 days of red-light therapy and the proportion of Ki67-positive cells (red fluorescence) relative to DAPI-stained cells (blue fluorescence) was recorded. Compared to HFs in the control group without light therapy, the proportion of Ki67-positive cells in the 650 nm light-treated group was significantly increased. These data indicate that 650 nm red light stimulates the HF and promotes hair growth in an *ex vivo* model.

Treatment with 650-nm red light postponed hair catagen transition in an *in vitro* culture model

The hair cycle plays an important role in AGA, and the percentage of HFs in the anagen phase was significantly decreased in AGA patients. Therefore, we investigated the hair cycle of cultured HFs treated with or without 650 nm red light. Macroscopic images of cultured HFs were obtained every other day and the hair cycle stage was recorded. HFs show a remarkably thin hair matrix, an oval dermal papilla, and low melanin levels in the early catagen phase. These changes occurred on day 6 in HFs in the control group, while HFs in the 650-nm light-treated group appeared to be in anagen for 8 days (Fig. 2A). Moreover, the hair cycle stage was macroscopically quantifiable on day 8. As shown in Fig. 2B, a greater percentage of organ-cultured anagen scalp HFs remained in the 650 nm light-treated group compared to the control group (45.8%±4.8% vs. 33.3%±6.8%), while there was a lower percentage of catagen HFs in 650 nm light-treated group compared to the control group (54.1%±4.8% vs. 66.6%±6.8%) on day 8. All data suggested that 650-nm red light treatment postponed HF catagen transition and prolonged the anagen stage.

Overview of gene expression and identification of differentially expressed genes

To explore the mechanisms by which 650 nm light treatment promoted HF stimulation and hair growth, we examined the genome-wide transcriptional changes in human HFs, with and without 650-nm light therapy by RNA-seq. After the stringent process of bioinformatic analysis, we found 728 DETs between the light-treated and untreated groups. Among them, 410 transcripts were upregulated, and 318 transcripts were downregulated by the treatment of 650 nm red light. The differential expression analysis and the unsupervised hierarchical clustering showed that the transcriptional changes induced by 650 nm red light treatment were consistent within the two groups and the DEGs tended to be upregulated after the treatment (Fig. 3A). The volcano plot (Fig. 3B) was generated from DEseq2 results, from which all nonzero read count transcripts were calculated and determined.

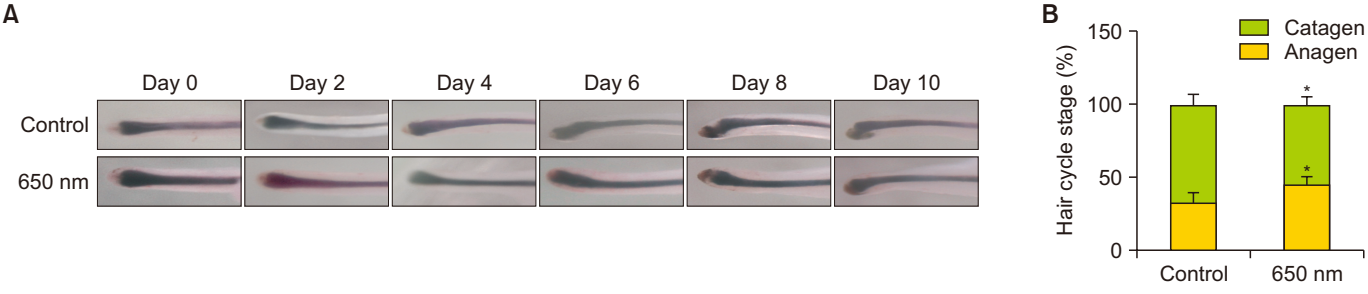

Fig. 2. Hair cycle stage detection in cultured human hair follicles. (A) Macroscopic images of cultured hair follicles were obtained every other day. (B) Macroscopic quantification of hair cycle stage on day 10 with 650-nm light treatment.

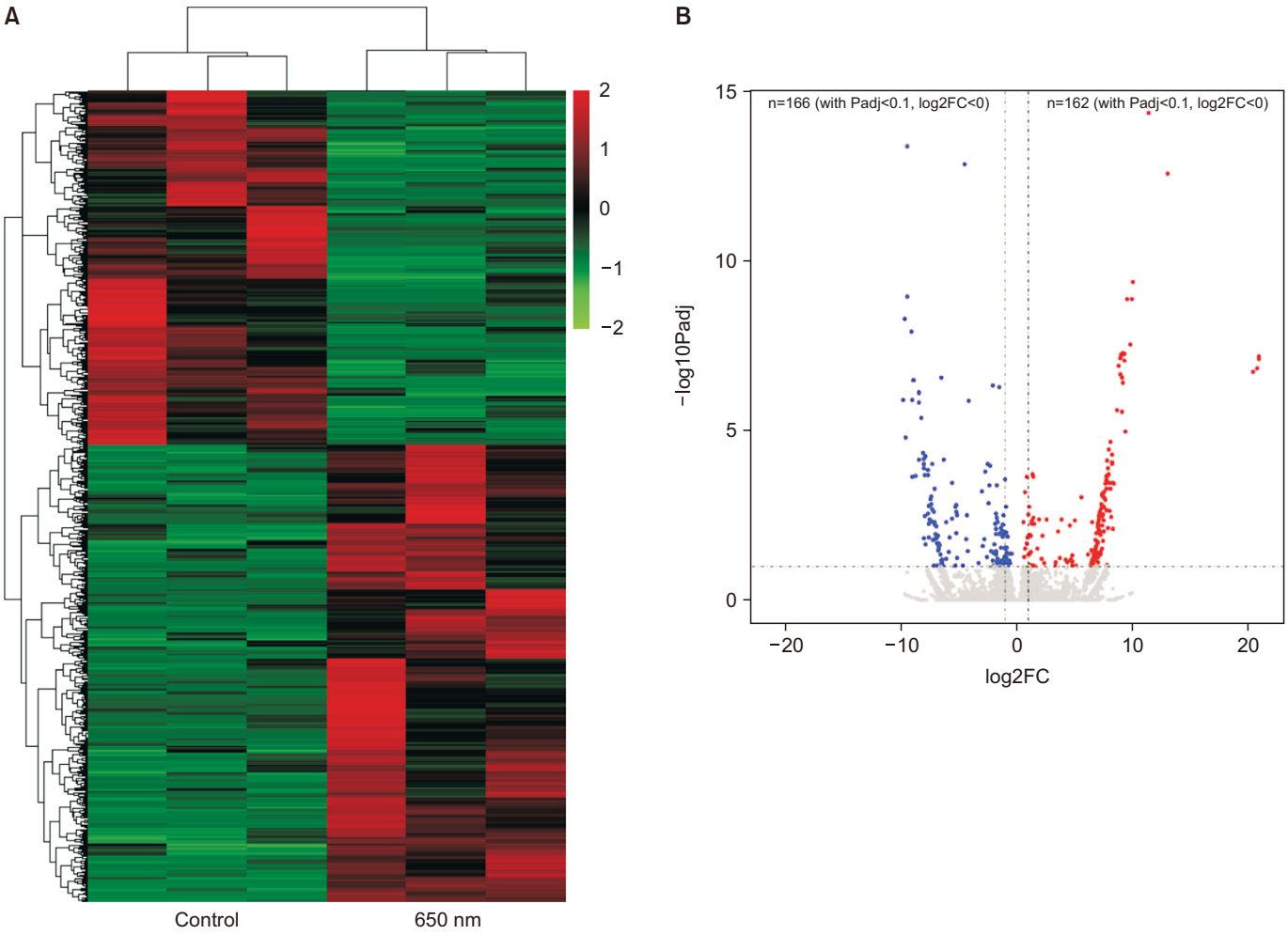

Fig. 3. Overview of gene expression and identification of differentially expressed genes. (A) The heatmap of all the differentially expressed transcripts between control and 650-nm light treatment groups. (B) The volcano plots generated from the results of DEseq2.

Transcriptome analysis of 650-nm red light-treated hair follicles

To further investigate the mechanism of 650-nm red light treatment, KEGG and GO analyses were performed using the top 500 DETs in the sleuth results. The top 15 enriched KEGG pathways are shown in Fig. 4A. Among them, the leukocyte transendothelial migration pathway, valine, leucine and isoleucine degradation pathways, the lysosome pathway, metabolic pathways, synthesis and degradation of ketone bodies pathway, and the adherens junction pathway are significant with a *p*-value <0.001. To further explore it, we verified the increased expression of transendothelial migration pathway related genes (*JAM3*, *CTNND1*, *RAP1B*, *ITGB2*), metabolism related genes (*PFKM*, *MAT1A*, *PHX2*, *GGT5*), and adhesion related genes (*MAP3K7*, *CTNNB1*, *FYN*, *CSNK2A1*) by RT-PCR. These results further confirmed the possible functional route of LLLT (Fig. 4C). GO analysis is composed of three parts: molecular function (MF), biological process (BP), and cellular component (CC). The results of GO analysis shown in Fig. 4B indicate that intracellular and intracellular parts are the most enriched MF pathways, while cell cycle and regulation of androgen receptor signaling are the most enriched BP pathways, and protein binding and ATP binding are the most enriched CC pathways. This work may lay a groundwork for further exploration of the mechanisms of HF stimulation via 650-nm red light treatment.

DISCUSSION

AGA is the most common form of hair loss in humans and is characterized by follicular miniaturization and progressive loss

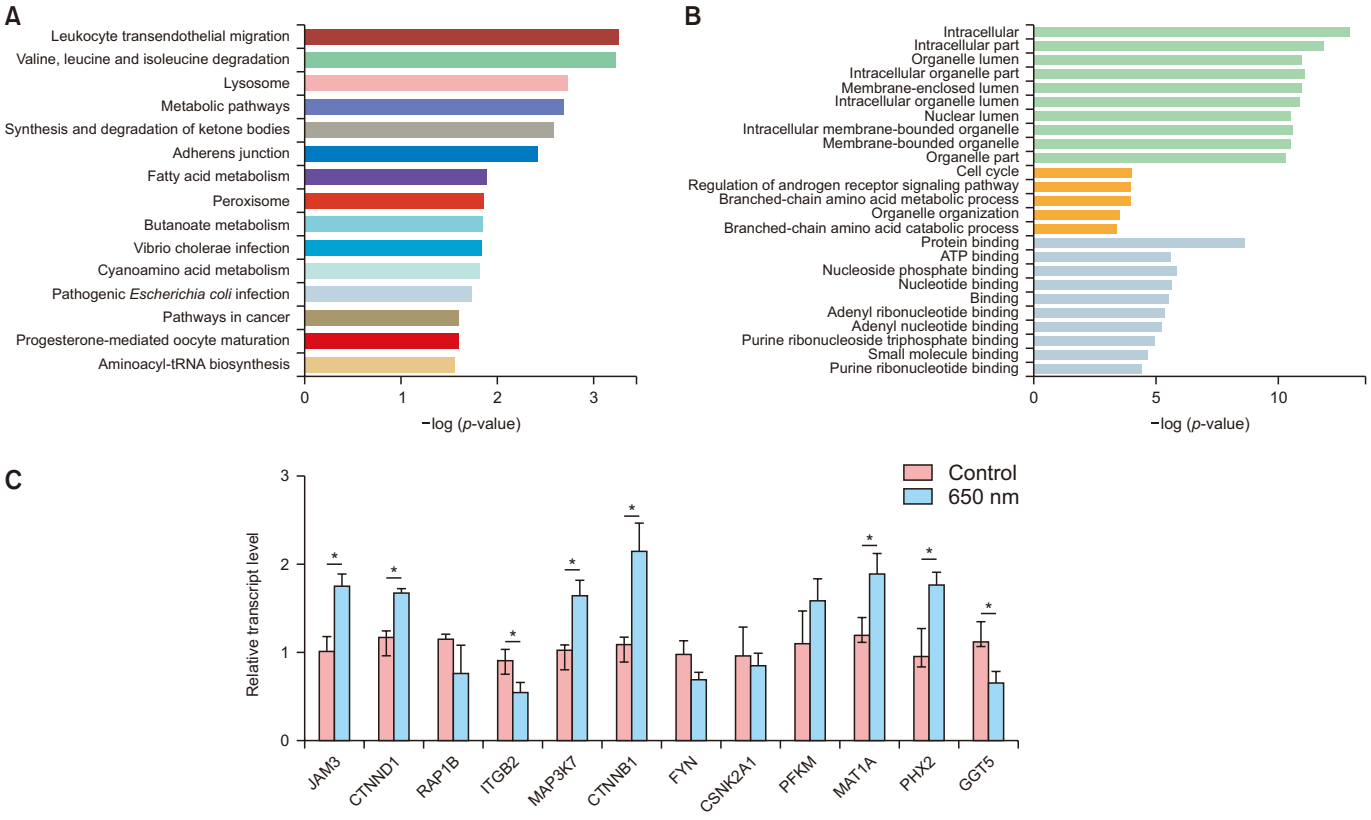

Fig. 4. Pathway enrichment analysis and gene expression detection. (A) The Kyoto Encyclopedia of Genes and Genomes pathway analysis of differentially expressed transcripts (DEGs). (B) Gene ontology analysis of DEGs. (C) The mRNA levels of related genes were detected by real-time PCR in hair follicles treated with or without 650-nm light.

of hair from the scalp. HF is a regenerating, hair shaft-producing mini-organ that undergoes cyclical periods of growth (anagen), regression (catagen), and relative quiescence (telogen). In the alopecic follicle, progressive decrease in the duration of anagen and reduction of the anagen to telogen ratio increase the proportion of telogen hairs. Telogen hair are more likely to shed, thus prolonging the lag period before generation of new hair shafts and reducing the number of visible hairs[20]. Previous studies revealed that *ex vivo* HF organ culture is an ideal model for research for AGA and other types of alopecia[21]. Therefore, we used cultured HFs to investigate the effects of 650 nm light therapy on AGA and its associated cellular mechanisms.

Among affected males and females, AGA has caused cosmetic problems leading to psychological distress. Topical minoxidil or finasteride as well as hair transplantation are effective standard and common treatments currently available in the clinic. Many previous studies demonstrated that LLLT has been used for a variety of treatments, such as wound healing, antibiosis, anti-inflammation, and immune modulation. Recently, LLLT has been used as an alternative and adjuvant treatment for patients for whom traditional medical therapy is not adequate or appropriate. LLLT has been approved by the FDA to treat male and female pattern hair loss via home medical devices. There have been a lot of *ex vivo*, animal, and clinical studies evaluating the effects of LLLT on hair loss[12], and it has been proposed that LLLT stimulates the growth of HFs, prolongs the anagen phase, and promotes anagen reentry[22]. Our present study also revealed that LLLT promotes human hair growth and prolongs the anagen phase in an *in vitro* HF culture model.

However, the mechanisms responsible for the efficacy of LLLT in the treatment of hair loss are not well understood. Previous studies indicated that 650-nm red light was the most effective and practical wavelength for stimulating hair growth via LLLT treatment. Therefore, we performed RNA-seq of 650-nm light-treated *ex vivo* HFs in this study. GO analysis of RNA-seq revealed that cell cycle and regulation of androgen receptor signaling are the most enriched BP pathways. Thus, LLLT can promote proliferation, which dependent on cell cycle. In addition,

androgen receptor signaling plays an important role in both cell cycle and hair growth of HFs[23]. Thus, we believe that LLLT can also regulate the cell cycle and androgen receptor signaling to confront AGA. Our data revealed that 650-nm light therapy promoted HF proliferation, suggesting that 650-nm light may promote human hair growth and prolong the anagen phase by regulating the cell cycle. KEGG analysis showed that several main biological pathways, such as the leukocyte transendothelial migration, amino acid degradation, lysosome, and metabolic pathways, were involved. Prior trials of LLLT have shown a decrease in inflammatory prostaglandin E-2 and an increase in inflammatory cytokines, such as IL-6, IL-8, TNF-α[24-26]. Our transcriptional data suggested that LLLT inhibits leukocyte migration and infiltration and may play an anti-inflammatory role in protecting HFs. In particular, the *CTNNB1*, *RAP1B*, *GNAI1*, *JAM3*, *CLDN5*, *VCAM1*, *CTNND1*, *CLDN18*, *ITGB2* genes are included in the leukocyte transendothelial migration pathway in KEGG. These genes are known to be expressed on the endothelium of blood vessels or leukocytes. The adherens junction is a key regulator of tissue architecture and dynamics via control of cell proliferation, motility, and survival[27]. During tissue inflammation, the adherens junction is disrupted[27]. Inflammatory processes, in turn, cannot do without endothelial cell adherens junctions[28]. Thus, our results may reveal that the two pathways interact with each other in the process of LLLT promoting hair growth. Besides, LLLT has been reported to increase metabolism of cell lines such as HeLa cells in the past[29]. Our results also found that its effect of promoting hair growth is related to the metabolic pathway and those metabolites can be produced or decomposed from acid degradation and lysosome pathways. Moreover, our real-time PCR results showed that 650 nm light treatment increased Wnt signaling pathway-related genes, such as Wnt10b and β-catenin (Supplementary Fig. 1). These data further indicated that the Wnt pathway, which is important in AGA, also played a role in the effects of LLLT treatment[30].

In summary, this study demonstrates that red light of 650 nm promoted human hair growth and inhibited spontaneous catagen transition in ex vivo HFs. Furthermore, transcriptome analysis suggested that 650 nm red light promoted the activation of HFs via regulating multiple signaling pathways, such as the leukocyte transendothelial migration pathway and amino acid degradation, which lays the groundwork for further exploration of red light and associated benefits via LLLT. Ultimately, our results strongly support the benefit of LLLT in the treatment of AGA.

SUPPLEMENTARY MATERIALS

Supplementary data can be found via http://anndermatol.org/src/sm/ad-33-553-s001.pdf.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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DATA SHARING STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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