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A novel liquid plasma derivative inhibits melanogenesis through upregulation of Nrf2

Ju Hyun Yun[1,5], Yeon Soo Kim[2,5], Hee Young Kang[3], Sung Un Kang[4,6]✉ & Chul-Ho Kim[4,6]✉

Non-thermal plasma (NTP) is an emerging technology with extensive applications in biomedicine, including treatment of abnormal pigmentation. However, very few studies have investigated how plasma induces anti-melanogenesis. Here, liquid plasma was prepared by treating an NTP jet with helium and oxygen (as carrier gases) for 15 min in serum-free culture media. In the zebrafish model, pigmentation ratio was observed with or without liquid plasma. The anti-melanogenic effect of liquid plasma was evaluated in human melanocytes by assessing the expression of melanogenesis-related genes using western blotting, RT-PCR, and immunohistochemistry. Liquid plasma reduced pigmentation in the zebrafish model and inhibited melanin synthesis in primary human melanocytes. Intracellular reactive oxygen species levels decreased and Nrf2 expression increased in liquid plasma–treated melanocytes. Liquid plasma affected microphthalmia-associated transcription factor (MITF) and tyrosinase mRNA and protein levels, tyrosinase activity, and melanin content. Considering the role of Wnt/β-catenin and PI3K/Akt pathways in melanogenesis, the effect of liquid plasma on this pathway was determined; liquid plasma decreased active β-catenin, LEF1/TCF4, MITF, and tyrosinase levels in a time-dependent manner and inhibited the nuclear translocation of β-catenin. This inhibition subsequently suppressed melanogenesis by downregulating *MITF* and *tyrosinase*. These results suggest that liquid plasma may be used for treating pigmentary disorders.

Keywords Liquid type non-thermal plasma (liquid plasma), Melanocytes, Melanin, β -catenin, *MITF*

One of the most actively explored realms in applied technology utilizing plasma is a medical-related study, called 'plasma medicine'[1]. Plasma, often referred to as the fourth state of matter alongside solid, liquid, and gas, is created by applying energy on a neutral gas, causing the electrical conductivity of the ionized gaseous material to improve over time[2,3]. Plasma includes charged ions, free electrons, reactive species, and fragmented molecules, and releases electromagnetic radiation, primarily in the form of UV radiation and visible light[4]. This unique state of matter is not only found in naturally occurring phenomena, such as stars, lightning bolts, and auroras, but is also used for various artificial applications in our daily lives, including fluorescent lights, neon signs, and plasma TVs[5]. Faure et al., developed a laser-plasma accelerator capable of generating monoenergetic electron beams, suggesting broad potential for applications in radiotherapy and radiobiology[6].

Non-thermal plasma (NTP) is a subtype of plasma, which is produced below 40 °C and under atmospheric pressure[7]. Due to its relatively low temperature, NTP does not harm tissues, and is therefore widely used in various medical fields. NTP can stimulate blood clotting by activating platelets and initiating fibrin formation[8]. Additionally, various reactive oxygen and nitrogen species are generated, including radicals, ozone, and peroxides. These species exhibit potent antimicrobial properties and effectively damage the cell membrane, proteins, and DNA of bacteria[9]. Some studies reported that plasma-induced reactive oxygen species (ROS) contribute to wound healing by promoting molecular signaling pathways associated with epithelial migration, inducing the secretion of cytokines, and stimulating collagen synthesis[10–12]. Notably, the alteration of cell adhesion properties and the induction of apoptosis by plasma have demonstrated its potential for use in cancer therapy[13–15].

Clinical applications of plasma are prevalent in the field of dermatology. Plasma skin regeneration, an innovative plasma-based energy device for skin treatments, has been used in the US since 2005, after receiving approval from the United States Food and Drug Administration. This device has shown promise for improving

[1]Department of Otorhinolaryngology-Head and Neck Surgery, College of Medicine, Ewha Womans University, Seoul 07985, Korea. [2]Department of Otorhinolaryngology-Head and Neck Surgery, Korea University College of Medicine, Seoul 02841, Korea. [3]Department of Dermatology, School of Medicine, Ajou University, Suwon 16499, Korea. [4]Department of Otolaryngology, Ajou University School of Medicine, Suwon 16499, Korea. [5]These authors contributed equally: Ju Hyun Yun and Yeon Soo Kim. [6]These authors jointly supervised this work: Sung Un Kang and Chul-Ho Kim. ✉email: cows79@ajou.ac.kr; ostium@ajou.ac.kr

pigmentation and skin texture by stimulating the regeneration of new collagen in the dermal layer[16]. Abnormal pigmentation, such as melasma, freckles, and age spots are closely linked to aging, and the growing interest in anti-aging care underscores the limitless development potential of such plasma-based devices[17]. However, the precise mechanisms underlying the influence of plasma on pigmentation remain unclear. Therefore, this study was performed to confirm whether liquid-type NTP (liquid plasma) can enhance anti-pigmentation and reveal how it inhibits melanogenesis.

Results

No cytotoxic effects on human primary melanocytes following exposure to liquid plasma

In this study, we investigated the anti-melanogenic effect of liquid plasma manufactured through a 4 kV discharge in 15 mL of serum-free medium for 15 min (Fig. 1A). Because liquid plasma does not induce cell death in normal cells, we examined its effect on melanocytes[11]. After treating melanocytes with liquid plasma for 1–3 days, we determined liquid plasma-induced cytotoxicity and melanocyte viability using the MTT assay. As shown in Fig. 1B, the liquid plasma had no cytotoxic effects on human primary melanocytes.

Inhibition of pigmentation in liquid plasma-cultured zebrafish and ex-vivo skin organ cultures

Zebrafish are useful animal models for observing pigmentation owing to their rapid growth and transparent bodies. To observe the anti-pigmentation effect of liquid plasma in vivo, we conducted a test using zebrafish. In Zebrafish, pigmentation was complete, and stripes appeared three days after hatching. Therefore, we cultured eggs in liquid plasma-treated water until hatching and evaluated the pigmentation grade at the indicated times. The pigmentation level was reduced by the liquid plasma treatment (Fig. 2A). It has been reported that changes in pigment patterns during zebrafish development are evident through changes in the distribution of melanophores[18,19]. Therefore, we quantified the melanophores distributed in liquid plasma-treated or untreated zebrafish. Samples were collected at defined positions in the lateral stripes. As shown in Fig. 2B, the control group had normal pigment and melanophore formation, but after LP treatment, pigment and melanophore development abnormalities appeared. Skin organs derived from human foreskin samples, were treated with liquid plasma for 3 days. Following this treatment, formalin-fixed, paraffin-embedded tissue sections were obtained from these skin organs to visualize melanin pigments. The ratio of pigmented area to epidermal area was significantly reduced after plasma treatment (Fig. 2C). These results indicated that liquid plasma inhibits in vivo and ex vivo pigmentation without damaging melanocytes or normal cells.

Inhibitory effect of liquid plasma on melanin synthesis in primary melanocytes

According to the results in Fig. 2, the anti-pigmentation effect of liquid plasma was determined. Therefore, we examined whether liquid plasma inhibited melanin synthesis in human primary melanocytes from the three donors. No morphological changes were observed after liquid plasma treatment (Fig. 3A). Tyrosinase is an important enzyme that catalyzes the first rate-limiting step in melanin synthesis, and microphthalmia-associated transcription factor (MITF) is a transcription factor involved in tyrosinase gene expression. MITF and tyrosinase protein levels decreased after liquid plasma treatment compared to normal conditions (Fig. 3B). The mRNA levels of *MITF* and *tyrosinase* were also decreased in melanocytes treated with liquid plasma (Fig. 3C). We conducted a tyrosinase activity assay and confirmed that liquid plasma significantly reduced tyrosinase activity (Fig. 3D). Because the expression and activity of tyrosinase were downregulated after liquid plasma treatment, a reduction in melanin content in melanocytes was observed (Fig. 3E). These results demonstrate that liquid plasma induces an anti-pigmentation effect by modulating the MITF-tyrosinase signaling pathways.

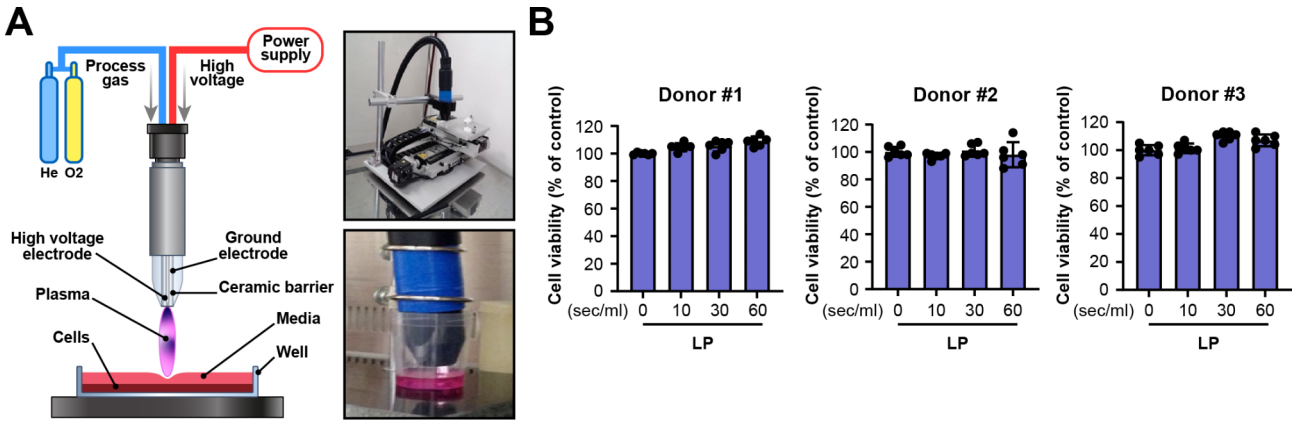


Fig. 1. Liquid plasma showed no cytotoxicity in human melanocytes. (**A**) Schematic diagram of the experimental setup for liquid plasma (**B**) Human primary melanocytes were treated with liquid plasma for 10, 30, and 60 s/mL, and viability was evaluated through MTT assay. LP, liquid plasma.

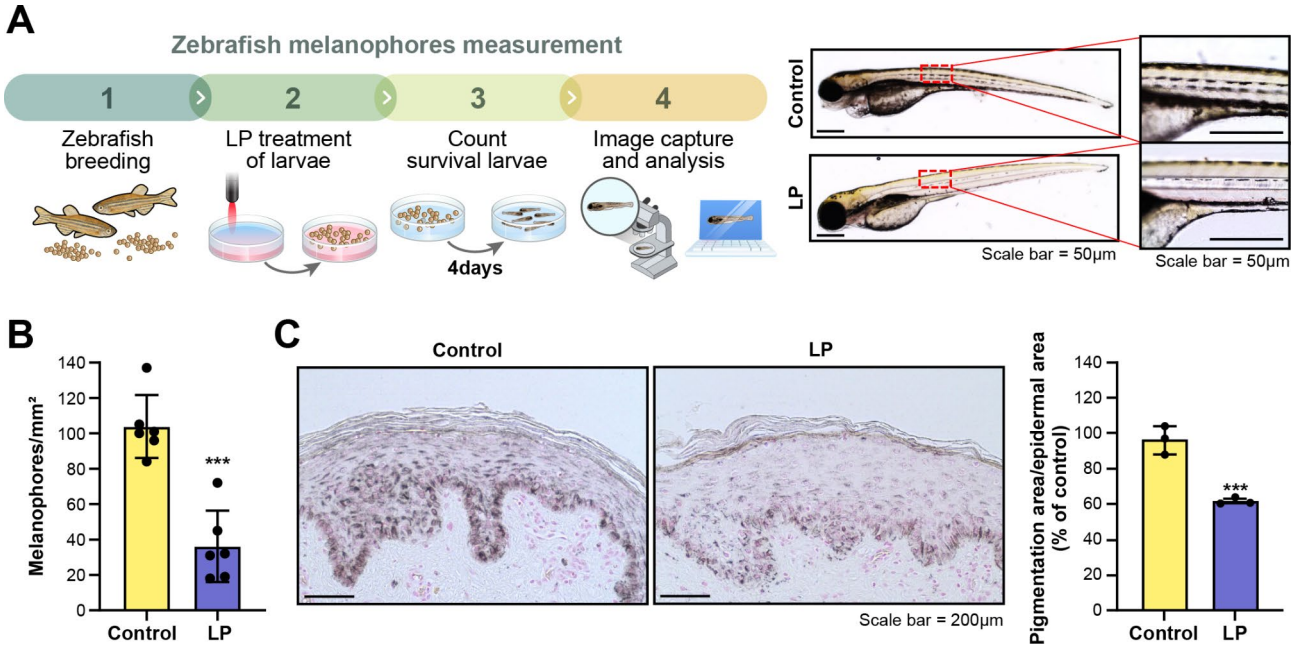


Fig. 2. Liquid plasma inhibits pigmentation in zebrafish and ex-vivo skin organ cultures. (**A**) The degree of pigmentation was greatly reduced in zebrafish whose larvae were cultured in liquid plasma-treated media for 24 h. (**B**) Melanophores were quantified in the control and liquid plasma-treated group. (**C**) Skin collected from human foreskin samples, was cultured with plasma treatment for 3 days. Fontana-Masson staining was performed on paraffin blocks of these cultured samples to visualize melanin pigments. The pigmented area per epidermal area was measured after image analysis. ***$p < 0.001$. LP, liquid plasma.

Suppression of melanogenesis by liquid plasma-mediated Nrf2 upregulation in a time-dependent manner

The Wnt/β-catenin signaling pathway is known for its relationship with melanogenesis[20]. β-catenin interacts with LEF1/TCF4, a transcription factor of the *MITF* gene and the stability and activity of LEF1/TCF4 depend on the phosphorylation status of β-catenin at serine 675 (Ser 675)[20–22]. Therefore, we investigated the phosphorylation status of β-catenin and the expression of several factors involved in melanogenesis by western blotting after 24 h of liquid plasma treatment. Liquid plasma treatment resulted in a time-dependent reduction in LEF1/TCF4 levels. In addition, the levels of Ser 675-phosphorylated β-catenin, MITF, and tyrosinase were decreased in a time-dependent manner after liquid plasma treatment (Fig. 4A). As shown in Fig. 4B, liquid plasma reduced MITF and tyrosinase gene expression as the post-treatment time increased. Since NTP produces various ROS and oxidative stress can affect Wnt signaling pathway activation, we measured H_2O_2 and ROS levels using a hydrogen peroxide assay kit and dihydroethidium[23]. Extracellular H_2O_2 levels significantly increased with increasing plasma-treatment time (Supplementary Fig. 1). However, significantly reduced ROS levels were observed in three human primary melanocytes (Fig. 4C). Ishii et al. reported that H_2O_2 was related to the rapid translation of Nrf2 and the induction of Nrf2's nuclear translocation[24]. Western blot analysis showed that the expression level of Nrf2 was increased, but the expression level of Keap1 was decreased (Fig. 4D). These results suggest that H_2O_2-induced Nrf2 activation upregulates antioxidant enzymes and protects melanocytes from oxidative stress. Furthermore, they suggest that liquid plasma can transcriptionally and post-translationally suppress the molecules involved in melanogenesis in a time-dependent manner.

Decreased nuclear translocation of phosphorylated β-catenin in liquid plasma-treated cells

Ser 675-phophorylated β-catenin, an active form of β-catenin, translocate into the nucleus and activates the transcription of its target gene, MITF[20]. Western blotting was conducted to examine the differences in the expression of melanogenesis-related proteins in the nucleus and the cytosol dependent on liquid plasma treatment after the nucleus is isolated from melanocytes. Liquid plasma reduced the active form of β-catenin in the nucleus, followed by suppression of MITF gene expression. Finally, the expression of tyrosinase in the nucleus was reduced, resulting in inhibition of melanogenesis (Fig. 5A). This was confirmed using immunocytochemical staining. β-catenin (green) was observed in the nuclei of cells untreated with liquid plasma. However, in cells treated with liquid plasma, β-catenin was primarily detected in the cytosol of melanocytes (Fig. 5B). These results suggest that liquid plasma reduces the nuclear translocation of phosphorylated β-catenin, which activates the process of melanogenesis.

Discussion

NTP has demonstrated intricate biological effects through direct interactions or indirect mechanisms involving various gases[25,26]. It can be used in various medical fields because it promotes blood clotting, bacterial killing,

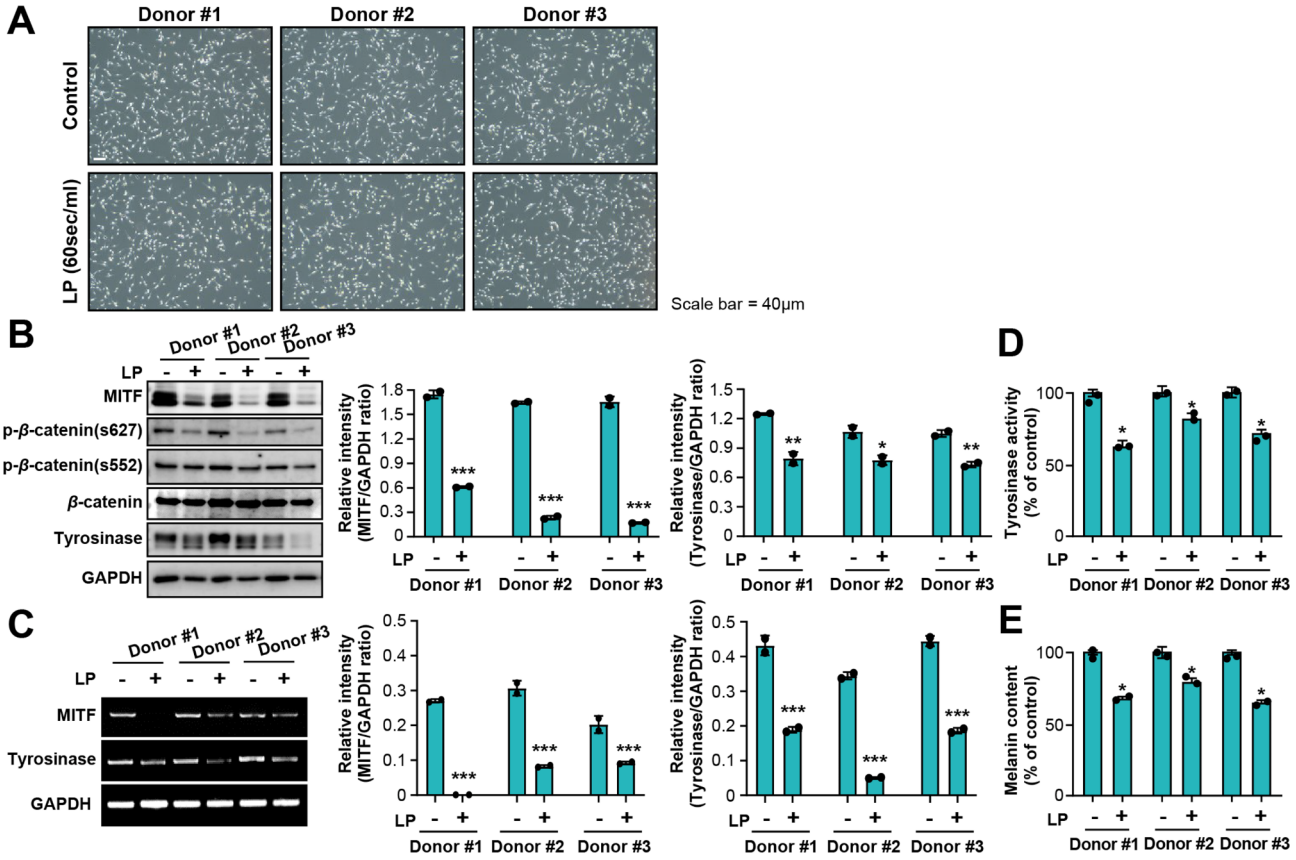


Fig. 3. Liquid plasma reduces melanin synthesis in primary melanocytes. (**A**) No morphological changes were observed in liquid plasma-treated and untreated primary melanocytes from 3 donors. (**B**) The expression of β-catenin, MITF, and tyrosinase was analyzed in liquid plasma-treated melanocytes by western blots. (**C**) The effects of liquid plasma on *MITF* and *tyrosinase* mRNA levels were evaluated by RT-PCR. (**D**) Tyrosinase activity was significantly reduced in melanocytes treated with liquid plasma. (**E**) Melanin contents were decreased after liquid plasma treatment. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. LP, liquid plasma; MITF, Microphthalmia-associated transcription factor.

wound healing, and causes death of cancer cells[8,9,13–15,27]. Recently, plasma-based energy devices have been commercially used in the beauty industry due to their effectiveness in mitigating abnormal pigmentation. However, these devices interact directly with the biological targets, precluding their storage and future use. Conversely, liquid plasma, achieved by activating a liquid medium with plasma, offers an indirect exposure approach that offers flexibility and the potential for the integration of anti-aging cosmetics[1]. Therefore, this study was conducted to explore whether liquid plasma could be used as an alternative to treat pigmentation disorders.

In contrast to previous studies, liquid plasma inhibits pigmentation in human primary melanocytes and zebrafish. According to Ali et al., liquid plasma generated from an N_2-feeding gas atmospheric pressure plasma jet, resulted in increased melanin content, tyrosinase activity, and *MITF* expression in melanomas[28]. Another study reported that NTP-activated medium (PAM) promotes melanogenesis by activating key enzymes such as tyrosinase and tyrosinase-related proteins (TRP1 and TRP2). PAM was generated by a microwave plasma device using argon gas[29]. In this study, liquid plasma, prepared by treating helium (He)/oxygen (O_2) plasma with culture media, downregulated the expression of melanogenesis-related molecules, including *MITF*. These differences are believed to be due to the types of gases used in creating the NTP. The type and proportion of reactive species and radicals generated by NTP depend on the choice of carrier gases. The proper concentration of H_2O_2 is important for activating Nrf2's antioxidant mechanism, which affects anti-melanogenesis[24]. NTP generated for N_2 gas does not produce H_2O_2[30]. A similar amount of ROS to 100 μm H_2O_2 treatment was detected in NTP generated from argon gas, implying that a high concentration of H_2O_2 from argon plasma in inhibits Nrf2-mediated antioxidant mechanism[29]. However, a lower concentration of H_2O_2 from He/O_2 plasma compared to argon plasma increased Nrf2 expression. Of course, further studies are needed to understand the molecular mechanisms of how NTP based on different types of gases produces different effects. Additionally, our previous study reported that NTP shows different effects depending on the cell type[11]. The study using an N_2-feeding gas atmospheric pressure plasma jet used B16 melanoma, unlike our study using human melanocytes form patient donors[28].

Nrf2 is considered a key molecule in the liquid plasma-induced anti-melanogenic effect. It is known that non-lethal levels of H_2O_2 increase Nrf2 synthesis and promote its nuclear translocation[24]. Once translocated to the nucleus, Nrf2 interacts with the antioxidant response element to enhance the expression of antioxidant

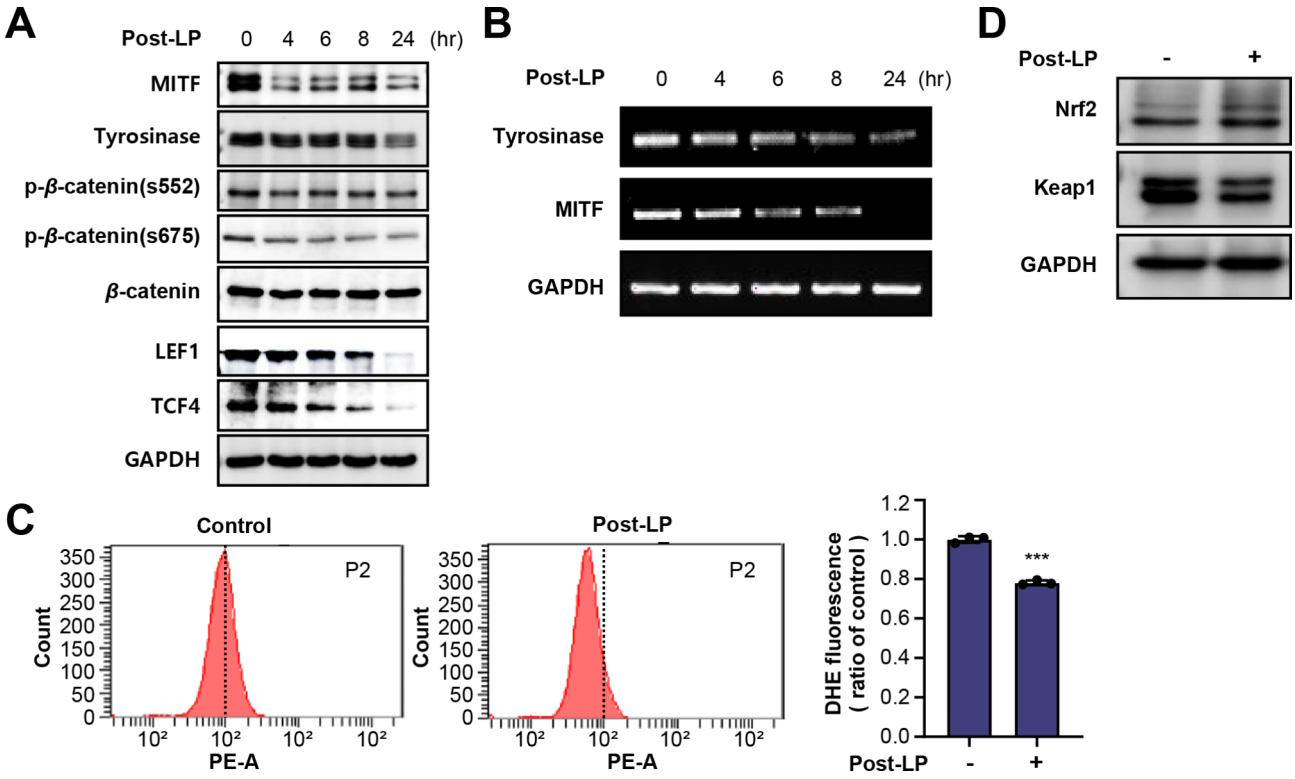


Fig. 4. Liquid plasma suppresses melanogenesis by regulating transcription factors of MITF through Nrf2 upregulation in a time-dependent manner. (**A**) Expression of MITF transcription factors was assessed by western blotting using antibodies against LEF1, TCF4 after 4, 6, 8, 24 h of liquid plasma treatment. (**B**) Downregulation of *MITF* and *tyrosinase* mRNAs was observed immediately after liquid plasma treatment. (**C**) To quantify ROS level, human melanocytes were treated with dihydroethidium, and these fluorescence-stained cells were analyzed using FACS. (**D**) Western blot analysis was conducted using anti-Nrf2 and Keap1 antibodies. ***$p < 0.001$. LP, liquid plasma; MITF, Microphthalmia-associated transcription factor.

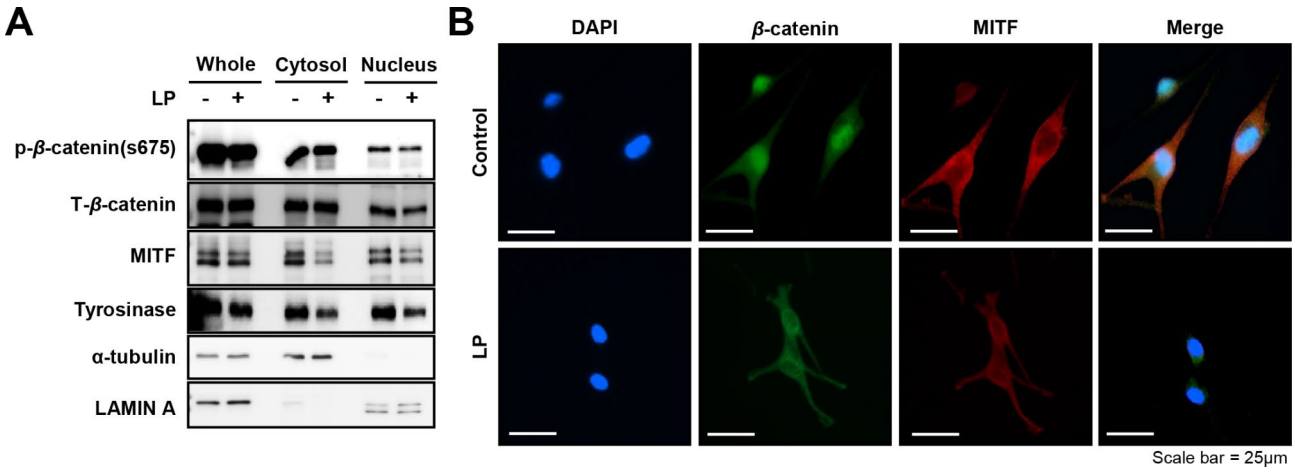


Fig. 5. Liquid plasma inhibits nuclear translocation of β-catenin. (**A**) The protein expression levels of Ser 675-phosphorylated β-catenin, MITF, and tyrosinase were evaluated in the nucleus and cytosol separately to determine the effect of liquid plasma on the nuclear translocation of β-catenin. (**B**) Immunocytochemical assay for β-catenin and MITF. LP, liquid plasma; MITF, Microphthalmia-associated transcription factor.

defense genes such as NQO1, HO-1, and GSTs, thereby playing a protective role against oxidative stress in cells[24]. Oxidative stress activates Wnt/β-catenin signaling pathways, inducing melanin synthesis[23]. MITF plays a crucial role in regulating melanin synthesis and melanosome biogenesis and transport[31]. The Wnt protein binds to Frizzled receptors, followed by the interaction between β-catenin and the LEF1/TCF4 transcription factor. This

interaction subsequently activates the *MITF*-M promoter, which is selectively expressed in melanocytes[32]. We demonstrated that liquid plasma downregulated the MITF protein, tyrosinase mRNA levels, melanin content, and tyrosinase activity in human primary melanocytes without causing toxicity (Fig. 3). The transcription factors and co-factors of MITF such as LEF1, TCF4, and β-catenin were also inhibited by liquid plasma treatment in a time-dependent manner (Fig. 4). Liquid plasma reduced the active form of β-catenin (Ser 675) in the nucleus, suggesting that it suppresses melanogenesis by inhibiting *MITF* gene expression (Fig. 5). Reduced intracellular ROS levels were measured and expression of Nrf2 was increased (Fig. 4). These results suggest that liquid plasma-induced Nrf2 activates the antioxidant system, resulting in anti-melanogenic effects by negatively affecting the Wnt signaling pathway.

Additionally, we hypothesized that Nrf2 affects the ubiquitin-dependent proteasomal degradation of MITF through AKT/PI3K signaling pathway. Post-translational regulation of MITF occurs through phosphorylation and ubiquitination, which influence its activity and stability[33]. MITF is phosphorylated by AKT at serine 510, leading to its degradation through ubiquitin-related proteolysis. Considering that Nrf2 activates AKT/PI3K signaling pathway, increased Nrf2 by liquid plasma-induced H_2O_2 might affect the degradation of MITF through AKT activation, resulting in inhibition of melanogenesis[24,34]. In other words, this study identified that liquid plasma regulates MITF not only at the transcriptional level but also post-translationally.

Although we have investigated the anti-melanogenic effect of liquid plasma in zebrafish models and ex vivo skin, several hurdles must be overcome for clinical practice. First, it is necessary to determine whether liquid plasma exhibits a skin-lightening effect at the organ level without causing cytotoxicity and is non-toxic to other organs. Additionally, further evaluation is required regarding dosage, frequency of use, and long-term effects.

In conclusion, we confirmed the anti-melanogenic effect of liquid plasma without cytotoxicity by downregulating the Wnt/β-catenin signaling pathway and upregulating AKT/PI3K signaling pathway through Nrf2-induced antioxidant mechanisms. Liquid plasma suppressed the phosphorylation of β-catenin, followed by a reduction in downstream proteins. We also observed that MITF, an important tyrosinase transcription factor, is modulated at both transcriptional and post-translational levels. (Fig. 6). This research is significant in that it highlights the potential use of liquid plasma for pigmentary disorders in the future.

Methods

Experimental NTP system specifications

The plasma device is designed and manufactured as an atmospheric pressure spray NTP system with a newly designed arc-free and anti-static plate to provide uniform NTP for biological research applications. The plasma source was equipped with a pair of electrodes made of Al_2O_3 (high-voltage and ground electrodes; dimension, 10×40 mm^2; distance between electrodes, 2-mm) whose direct contact with the plasma was prevented by a ceramic barrier. The specifications of the power supply with this system are a minimum of 2 kV, a maximum of 13 kV, and a mean frequency of 20–30 kHz, which may vary depending on the type and amount of gas used. In this study, He and O_2 were used as carrier gases because we have previously shown that adding O_2 to He plasma improves the efficiency of cancer cell inhibition[35,36]. The voltage and current of the NTP were measured uniformly and stably. The plasma density using $He + O_2$ as a carrier gas was calculated to be approximately 106/ m^3 based on optical emission spectroscopy, and the ROS density was around 1013/ m^3. The temperature of the plasma gas was kept low at ~35 °C, even after 10 min at 13 kV for NTP treatment. The distance between the plasma device and the bottom of the culture dish was kept at approximately 2 cm. The NTP jet partially dispersed the media.

Liquid plasma preparation

The method used to prepare the liquid plasma was optimized by testing under several different conditions and varying factors, such as distance to the media or treatment time. A protocol for producing liquid plasma has been established by detecting ozone or UV-A or UV-B from liquid plasma. Finally, liquid plasma was prepared with 15 mL culture medium. NTP was employed for 15 min in culture media, at a distance of approximately 1–2 cm. The prepared liquid plasma samples were measured as described in our previous study, and then the liquid plasma was used to treat the melanocytes at a constant concentration[37].

Cell culture

Normal human melanocytes were obtained from foreskin samples; they were maintained in a F12 medium supplemented with 10% fetal bovine serum (FBS, Gibco-BRL, Bethesda, MD), 24 μg/mL 3-isobutyl-1-methylxanthine, 80 nM 12-O-tetradecanoyl phorbor 13-acetate (TPA), 1.2 g/mL basic fibroblast growth factor (bFGF), and 0.1 μg/mL cholera toxin (Sigma, St. Louis, MO). For experiments, passage 2 or passage 7 melanocytes were maintained in medium 254 (Casade Bioogics) supplemented with a commercial growth factor cocktail (HMGS, consisting of 10 ng mL^{-1} phorbol 12-myristate 13-acetate (PMA), 3 ng mL^{-1} human recombinant basic fibroblast growth factor, 3 μg mL^{-1} heparin, 500 nM hydrocortisone, 5 μg mL^{-1} insulin, 5 μg mL^{-1} transferrin, 0.2% (v/v) bovine pituitary extract, and 0.5% (v/v) fetal bovine serum) at 37 °C in a humidified atmosphere containing 5% CO_2.

Cell viability assay

Melanocytes were seeded at a density of 5×10^3 cells/well in 96-well plates. After 1 d of culture, the effect of liquid plasma treatment on cell viability was analyzed for 24 h using an assay based on the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma Aldrich). Briefly, after adding MTT to the cell suspension (40 μL) for 4 h, the remnant formazan product was dissolved in 100 μL DMSO. The optical density of each well was measured at 540 nm using a microplate reader (Bio-Tek, Winooski, VT, USA). The results are presented as percentages relative to control cells.

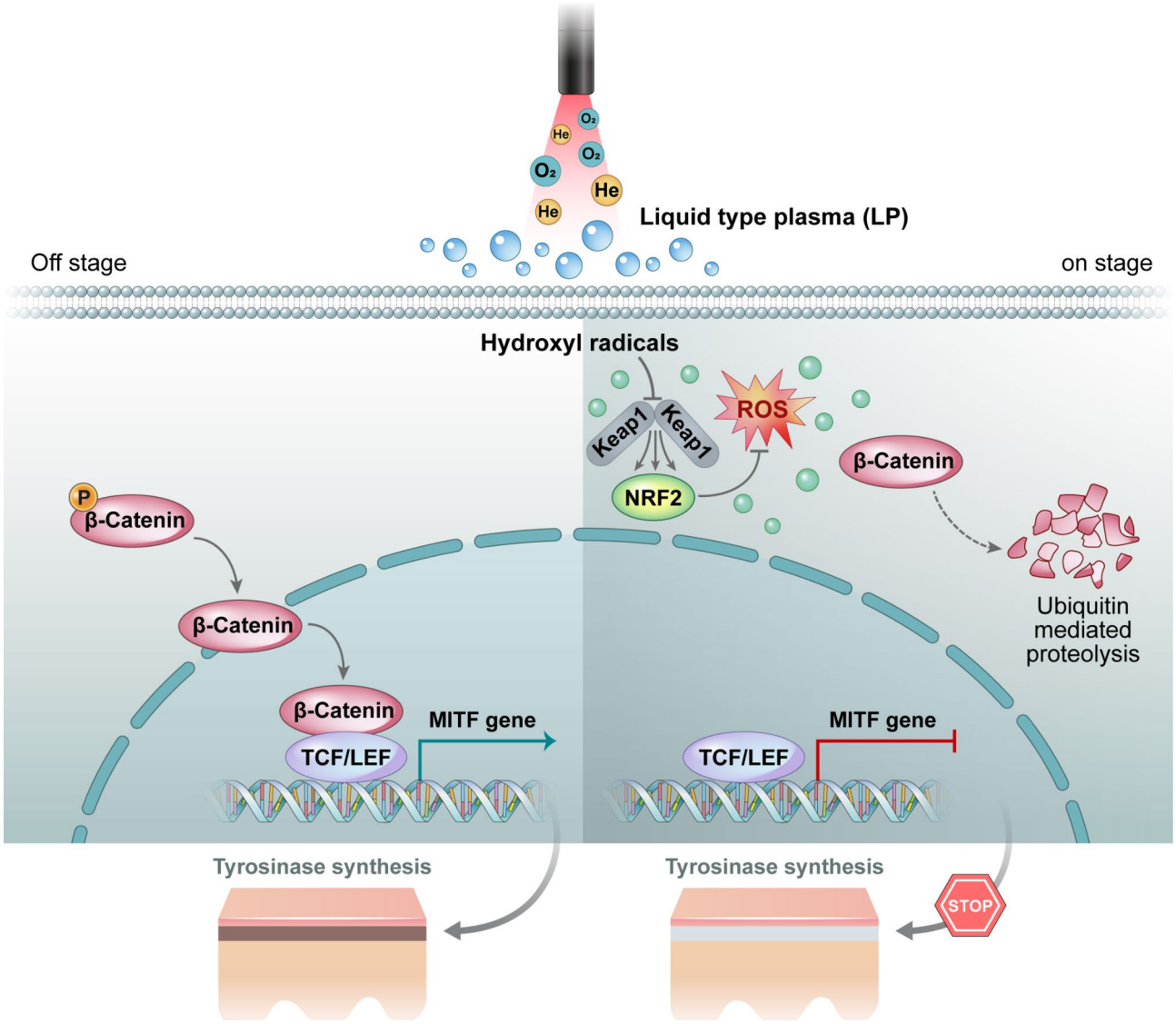


Fig. 6. Simplified scheme of liquid plasma-induced anti-melanogenesis. Hydroxyl radicals induced by liquid plasma, upregulated Nrf2 in melanocytes. Intracellular ROS levels were reduced through Nrf2-mediated antioxidant mechanisms. This reduction inhibited the phosphorylation of β-catenin, leading to the suppression of its nuclear translocation and promotion of its ubiquitin-mediated proteolysis. Consequently, this inhibition suppressed melanogenesis by downregulating MITF and tyrosinase synthesis.

In vivo zebrafish model and melanophore measurement

Animal care and experimental procedures adhered to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The protocols of this study received approval from the Committee for Ethics in Animal Experiments at Ajou University School of Medicine and all methods are reported in accordance with ARRIVE guidelines. Embryos of the AB wild-type strain Zebrafish (*Danio rerio*) were produced by paired mating adult fish at 28.5 °C in a 14 h light/10 h dark cycle. The embryos were maintained in 100 mm^2 Petri dishes in embryo media (1 mM $MgSO_4$, 120 µM KH_2PO_4, 74 µM Na_2HPO_4, 1 mM $CaCl_2$, 500 µM KCl, 15 µM NaCl, and 500 µM $NaHCO_3$ prepared in deionized H_2O [dH_2O]) at a density of approximately 50 embryos per dish. Four days post-fertilization (dpf), the larvae were fed dehydrated paramecia. Then, 2mL LP was treated in embryo medium in a 6-well plate. A total of 15 zebrafish larvae (4 dpfs) were treated with liquid plasma for 24 h. Zebrafish were mounted with methylcellulose on a depression slide for fluorescence microscopy. Melanophores were counted using Image J (National Institutes of Health, NIH, Maryland, USA).

Ex vivo skin organ culture and pigmentation assay

All experiments were approved by the Institutional Review Board of Ajou University Hospital (Approval no.: AJOUIRB-SMP-2017-438; approval date 2018-02-22) and performed in accordance with relevant guidelines and regulations. After obtaining informed consent, human foreskin samples were collected during surgery and cultured as described previously[38]. A sterilized stainless-steel grid was placed on a 35 mm culture dish.

Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% FBS was used as the filler medium for the stainless-steel grid. The skin specimens were placed on a stainless-steel grid and cultured in an incubator at 37 °C in a humidified atmosphere containing 5% CO_2. The skin organs were treated with liquid plasma. After 3 d, the specimens were fixed with 10% formalin and embedded in paraffin. Melanin pigments were visualized by Fontana-Masson staining. Image analysis was performed using Image Pro Plus Version 4.5 (Media, U.S.A.) and the pigmented area per epidermal area was measured.

Melanin content determination

Melanocytes were treated with liquid plasma for 24 h. The melanin content was measured according to the method described by Tsuboi et al. with a light modification[39]. Pellets of 2×10^5 cells were solubilized in 1 M NaOH and optical densities were measured at 490 nm using an enzyme-linked immunosorbent assay reader. The absorbance was estimated using a standard curve plotted using synthetic melanin (Sigma Chemical Co., St. Louis, MO, U.S.A.).

Tyrosinase activity assay

Dopa oxidase activity was determined using the method described by Tomita et al.[40]. Cells were solubilized in 1% Triton X-100 and 10 mmol/L L-DOPA (Sigma Chemical Co.). After 90 min incubation at 37 °C, the absorbance was measured at 490 nm.

Immunocytochemistry

Melanocytes were cultured on a microscope cover glass (Thermo Fisher Scientific, Rochester, NY, U.S.A.), and the cells were treated with liquid plasma for 24 h. After 24 h, the cells were fixed with 4% formaldehyde and blocked with 5% bovine serum albumin (BSA) in prepared in PBS for 45 min. The cells were then incubated overnight with a rabbit polyclonal anti β-catenin (1:500, Cell Signaling). After washing, cells were incubated with Alexa 488- or Alexa 546-conjugaed secondary antibody for 2 h at 20–22 °C. The nuclei were stained with Hoechst 33,345 (Invitrogen, Waltham, MA, U.S.A.). Image analysis was performed using EVOS FL Auto (Thermo Fisher Scientific, Carlsbad, CA, U.S.A.).

Western blotting

Melanocytes were lysed in RIPA buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1.0% nonidet-P 40 (NP40), 1.0% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS); Thermo Fisher Scientific, Rochester, NT, U.S.A.) supplemented with protease inhibitor cocktail and phosphoSTOP (Roche Applied Science, Vienna, Austria, pH 7.4). The proteins obtained from the lysates were run on 8% SDS-polyacrlamide gels and then transferred onto membranes before incubation with various antibodies at 20–22 °C for 2 h or overnight at 4 °C.

The following antibodies were used: anti-MITF, anti-LaminA (1:1000, Abcam, Cambridge, MA), anti-Tyrosinase (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), anti-β-catenin, anti-phospho-β-catenin (Ser 552, and 675), anti-LEF1, anti-TCF4, anti-Myc, and anti-GAPDH (1:1000, Cell Signaling Technology, Danvers, MA).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

MITF, tyrosinase, and *GAPDH* expression levels were estimated by RT-PCR. Total RNA was isolated from human melanocytes using TRIzol reagent (Invitrogen). cDNAs was synthesized using 5 μg total RNAs and ReverTra Ace® qPCR RT Master Mix (TOYOBO Co. Ltd, Japan) according to the manufacturer's instructions. The PCR primer sequences were as follows:

MITF sense 5′-CACATACAGCAAGCCCAA-3′.
antisense 5′-CAGTGCTCTTGCTTCAGA-3′.
tyrosinase sense 5′-ATCCAGAAGCTGACAGGA-3′.
antisense 5′-TTTGAGAGGCATCCGCTA-3'.
GAPDH sense 5′-GAGTACGTCGTGGAGTCCA-3′.
antisense 5′-ATGGCATGGACTGTGGTCA-3′.

PCR products were separated on a 1% agarose gel and observed using a LAS4000 (Fuji, Japan).

Measurement of extracellular H_2O_2

To measure extracellular H_2O_2 production, the plasma-treated supernatant was used with a hydrogen peroxide assay kit (Abcam, Cambridge, UK, ab102500) according to the manufacturer's protocol. The concentration of H_2O_2 in the supernatant was determined using a microplate reader (Epoch 2; BioTek Instruments, Inc., Winooski, VT, USA) with excitation at 540 nm. according to the manufacturer's protocol.

Measurement of intracellular ROS

To detect intracellular ROS production, cells were treated with liquid plasma for 24 h and then hydroethidine was used as described previously[41]. Fluorescent cells were analyzed using flow cytometry.

Statistical analysis

All data were compared using one-way analysis of variance (ANOVA) (SPSS 12.0; SPSS Inc., Chicago, IL, U.S.A.). The results are expressed as mean ± standard deviation (SD). All *p*-values were two-tailed, and *p*-values less than 0.05 were considered statistically significant.

Data availability

All data generated or analyzed during this study are included in this published article and its Supplementary Information files.

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Author contributions

J.H.Y. designed the study, analyzed the results, and wrote the manuscript; Y.S.K. analyzed the results and wrote the manuscript; H.Y.K. analyzed the results and wrote the manuscript; S.U.K. designed and performed all experiments and wrote the manuscript; C-H.K. supervised experiments and wrote the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to S.U.K. or C.-H.K.

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