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Anti-Hair Loss Effects of the DP_2 Antagonist in Human Follicle Dermal Papilla Cells

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Abstract: Prostaglandin D_2 (PGD_2) levels are high in the balding areas of human scalps, and PGD_2 has been found to inhibit hair growth. It is known that the inhibition of the PGD_2 receptor can promote hair growth by preventing hair follicles from entering the catagen phase. Thus, we identified an antagonist of DP2, the receptor for PGD2, as a potential treatment for hair loss using an AI-based DeepZema® drug development program. In this study, we identified that the DP_2 antagonist (DP2A) could ameliorate alopecia in human follicle dermal papilla cells (HFDPCs) that were stimulated by dihydrotestosterone (DHT), a known molecule related to hair loss. We observed that the DP2A promoted wound healing efficiency and increased alkaline phosphatase levels in the HFDPCs that were damaged with DHT. In addition, we found that the DP2A diminished the reactive oxygen species (ROS) levels generated in the DHT-damaged HFDPCs. We confirmed that the DP2A effectively recovered the membrane potential of mitochondria in these cells. We also demonstrated that the DP2A enhanced the phosphorylation levels of both Akt and ERK in the HFDPCs that were damaged with DHT. Notably, we revealed that the DP2A slightly enlarged the three-dimensional spheroid size in these cells and confirmed that the DP2A improved hair growth in the organ culture of human hair follicles. Taken together, we suggest that DP2A has therapeutic effects on HFDPCs that are damaged by DHT and holds promise as a potential treatment for treating hair loss.

Keywords: DP_2 antagonist; human follicle dermal papilla cells; hair growth; hair loss; dihydrotestosterone

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1. Introduction

Androgenetic alopecia (AGA) is men's most common pattern of hair loss, characterized by the gradual conversion of thick terminal hair into fine vellus hair [1]. The pathophysiological alterations in AGA are disruptions in the dynamics of hair follicles, especially the gradual shortening of the anagen phase. The 5-alpha reductase is a key enzyme to these changes that converts testosterone (T) into dihydrotestosterone (DHT) in dermal papilla cells. The strong binding of DHT to androgen receptors (ARs) initiates a signaling pathway that interferes with hair growth, resulting in hair follicle miniaturization [2,3]. Although extensive studies have been performed, the underlying mechanisms causing AGA are not fully understood.

Human follicle dermal papilla cells (HFDPCs), which are primarily used for studying the improvement of human hair loss, are mesenchymal cells that connect to capillaries

beneath the hair follicle [4,5]. These cells have stem cell capabilities and play a key role in stimulating hair growth. Hair growth is related to cell division and migration around the dermal papilla.

Previous studies have shown higher expression levels of prostaglandin D_2 synthase (PTGDS), which produces prostaglandin D_2 (PGD_2), in the balding scalp of patients with AGA. In addition, PGD_2 suppresses hair growth in both mouse and human models by interacting with the DP2 receptor [6,7]. The effects of PGD_2 occur through prostaglandin receptor 1, called DP1, or prostaglandin receptor 2, called DP2 [8,9]. An increase in androgen levels may lead to the production of PGD_2, as androgens stimulate PTGDS [10]. Previous reports demonstrated that PGD_2 facilitates the initiation of the catagen phase, resulting in the miniaturization of hair follicles. In addition, PGD_2 blocks hair follicle regeneration during wound healing [11,12]. These findings emphasize that PGD_2 affects hair growth.

Thus, we identified a DP2 antagonist (DP2A) using our AI-based DeepZema® drug development program [13] and examined if the DP2A could ameliorate HFDPCs that were damaged with DHT and serve as a potential agent for enhancing hair growth.

2. Materials and Methods

2.1. Chemicals and Reagents

The DP2A (Biphenylmethyl Carboxymethyl Benzyldimethylpyrazole Carbamate), was screened from a chemical library using an AI-based DeepZema® drug development program (Innovo Therapeutics Inc., Daejeon, Republic of Korea). The 2-propanol, dimethyl sulfoxide (DMSO), dihydrotestosterone (DHT), and minoxidil were acquired from Sigma (St. Louis, MO, USA).

2.2. Cell Culture

The HFDPCs (Promo Cell, Heidelberg, Germany) were grown in a cell growth medium. At 80% confluence, every 3 days, the cells were detached using a DetachKit containing a trypsin/EDTA ratio of 0.04%/0.03% and reseeded into a fresh 75 mm flask.

2.3. Cell Viability Assay

The HFDPCs were cultured in a 96-well plate. The cells were administered with each concentration of the DP2A for 24 h, respectively. Then, the culture medium was washed, and 10 µL of the reaction reagent of the EZ-cytox assay kit (DoGenBio, Seoul, Republic of Korea) and 100 µL of cell growth medium were treated and incubated for 1 h. The absorbance of each sample was assessed at 450 nm.

2.4. Wound-Healing Assay

The HFDPCs were cultured in a 6-well dish. After 24 h, a horizontal scratch was conducted across the center of the dish. After washing the medium, fresh culture media containing 5 µM DHT, each concentration of the DP2A, and 1 µM minoxidil were added to the wells. The cells were incubated for 24 h. An image of each sample was taken at 0 h and 24 h under a microscope.

2.5. Alkaline Phosphatase (ALP) Staining Assay

The HFDPCs were cultured in a 24-well plate. Each sample was added with 1 µM DHT, 5 µM DP2A, and 1 µM minoxidil, followed by 24 h incubation in a 5% CO_2 incubator. The alkaline phosphatase staining kit (purple) was acquired from Abcam (Cambridge, UK). The cells were treated with PBS-T for washing and added to a solution for fixation for 2 min. They were stained with an alkaline phosphatase solution for 24 h and washed with DPBS. The stained colonies were calculated using a Nikon phase contrast microscope (Tokyo, Japan).

2.6. DCF-DA ROS Assay

The HFDPCs were cultured in a confocal dish. After 24 h, the cells were administered with 1 μM DHT, 5 μM DP2A, or 1 μM minoxidil, followed by 24 h incubation in a 5% CO_2 incubator. The cellular ROS assay kit was purchased from Abcam (Cambridge, UK). The cells were treated with DPBS, for washing two times and stained with 2,7-Dichlorofluorescin diacetate (DCF-DA) for 15 min. After staining, the cells were treated with DPBS for washing, and 200 μL of DPBS was added. The fluorescence was then assessed using a Nikon Eclipse Ti2 fluorescence live-cell imaging microscope (Tokyo, Japan).

2.7. Mitochondrial Membrane Potential Measurement

The HFDPCs were cultured in a confocal dish (SPL, Gyeonggi-do, Republic of Korea). After 24 h, the cells were each administered with 1 μM DHT, 5 μM DP2A, and 1 μM minoxidil for 24 h. A mitochondrial membrane potential detection assay kit, known as JC-1, was obtained from Abcam (Cambridge, UK). The cells were administered with DPBS for washing twice and administered with 5 μM JC staining solution for 15 min. After washing with DPBS, 200 μL of DPBS was added for the measurements. The fluorescence was assessed using a Nikon Eclipse Ti2 fluorescence live-cell imaging microscope (Tokyo, Japan).

2.8. Western Blot Analysis

The HFDPCs were cultured in a 100 mm dish. After 24 h, the cells were administered with DPBS for washing two times and lysed using an RIPA buffer. The protein concentration was assessed by using a BCA assay. Next, 30 μg proteins for phosphor-/total AKT, phosphor-/total ERK, β-catenin, and β-Actin were resolved by SDS-PAGE. The gel was transferred to the PVDF membrane (Roche, Mannheim, Germany) and blocked with 3% non-fat dry milk. After that, the membrane was incubated with primary antibodies (p-AKT and AKT) (Cell Signaling Technology, Beverly, MA, USA) or primary antibodies (p-ERK and ERK) (Cell Signaling Technology, Beverly, MA, USA), and β-catenin (Santa Cruz Biotechnology, Dallas, Texas, USA) for 1 h, followed by TBS-T (Bio-Rad Inc., Hercules, CA, USA) for three washes. Then, secondary antibodies were applied for 1 h. The membranes were treated with TBS-T for washing. Each band was detected using an ECL reagent (Cytiva, Marlborough, MA, USA), and images were obtained with the Invitrogen iBright 1500 system and analyzed using Fiji Image J (Win 64-bit) software.

2.9. Spheroid Culture of HDPCs

The HFDPCs were cultured in 96-well microplates (Corning (Glendale, AZ, USA)) for 3 dimensions. The cells were treated with 1 μM DHT, each concentration of the DP2A, and 1 μM minoxidil. Each spheroid's diameter was assessed by a Nikon phase contrast microscope (Nikon, Japan).

2.10. Human Hair Follicle Organ Culture

Human scalp skin was acquired from patients' non-balding areas. This clinical study was approved by the Dankook University Hospital's Institutional Review Board (IRB no. DKUH. 2021-12-005) [13]. The human hair follicles chosen for anagen VI hair follicles were isolated through microdissection. Each treatment group (6 hair follicles) was repeated three times. The hair follicles were grown in William's E medium, containing L-glutamine (2 mM), penicillin (10 U/mL), streptomycin (100 μg/mL), hydrocortisone (10 ng/mL), insulin (10 μg/mL), and amphotericin B (25 μg/mL) and incubated in a 5% CO_2 incubator.

2.11. Statistical Analyses

The statistical analyses were processed by GraphPad Prism 5.0 with Tukey's Multiple Comparison Test being applied following a one-way analysis of variance (ANOVA). The data are indicated as the mean ± standard deviation (SD). A statistical significance was defined as a *p*-value of less than 0.05.

3. Results

3.1. The DP2A Enhanced Cell Viability in HFDPCs

To analyze the cytotoxicity of the DP2A in the HFDPCs, an MTT assay was performed. The results showed that the DP2A did not exhibit any cytotoxic effects on the HFDPCs. Additionally, the DP2A enhanced cell viability compared to the control (Figure 1), meaning that the DP2A can increase cell proliferation.

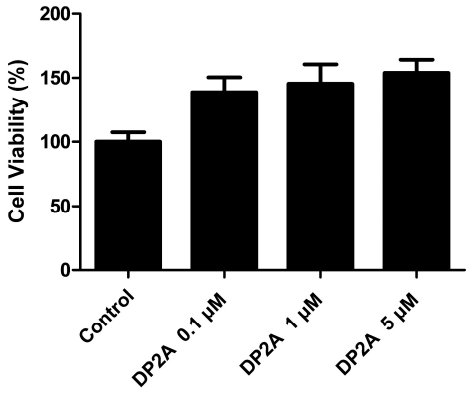


Figure 1. The cell viability of the HFDPCs with the DP2A.

The HFDPCs viability with the DP2A was measured using the MTT assay. The % of viable cells compared to the control was calculated. The data are presented as mean ± SD (n = 4).

3.2. The DP2A Improved the Migration of DHT-Damaged HFDPCs

A wound-healing assay was conducted on the HFDPCs to examine the wound-healing efficiency of the DP2A. The HFDPCs were scratched and treated with 5 μM DHT, various concentrations of the DP2A (0.1, 1, or 5 μM), and 1 μM minoxidil [14]. After 24 h, the DP2A increased the wound-healing regions of the DHT-damaged HFDPCs (Figure 2).

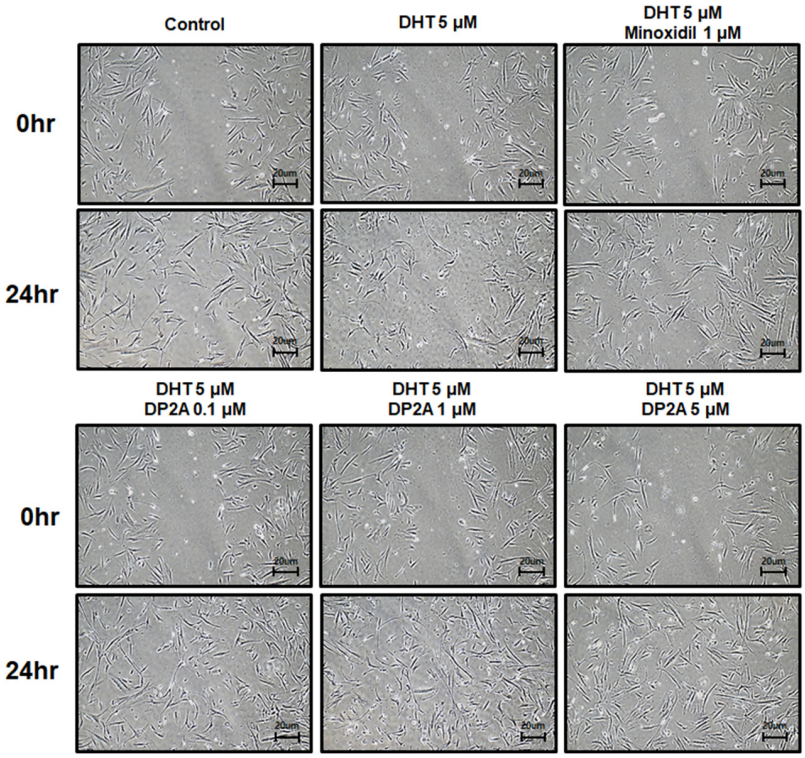


Figure 2. The wound-healing efficiency of the DP2A in the HFDPCs stimulated with DHT.

A wound-healing assay was conducted on the HFDPCs that were stimulated by 5 μM DHT. The cells were each treated with 0.1, 1, or 5 μM DP2A and 1 μM minoxidil for 24 h. Wound-healing images were acquired using a microscope. Each image was a representative of three independent experiments.

3.3. The DP2A Enhanced the Alkaline Phosphatase Level in the HFDPCs Damaged by DHT

Alkaline phosphatase is crucial for tissue regeneration. Its activity is observed in tissues undergoing remodeling or in regions with high cellular metabolic rates [15]. The activity of alkaline phosphatase is closely related to hair growth. A previous study indicates that minoxidil elevates alkaline phosphatase levels, promoting improved hair growth [16]. As expected, treatment with 1 μM minoxidil increased the alkaline phosphatase expression levels in the HFDPCs that were stimulated by DHT. Similarly, 5 μM DP2A enhanced the alkaline phosphatase levels in the HFDPCs that were damaged with DHT in comparison to the control (Figure 3).

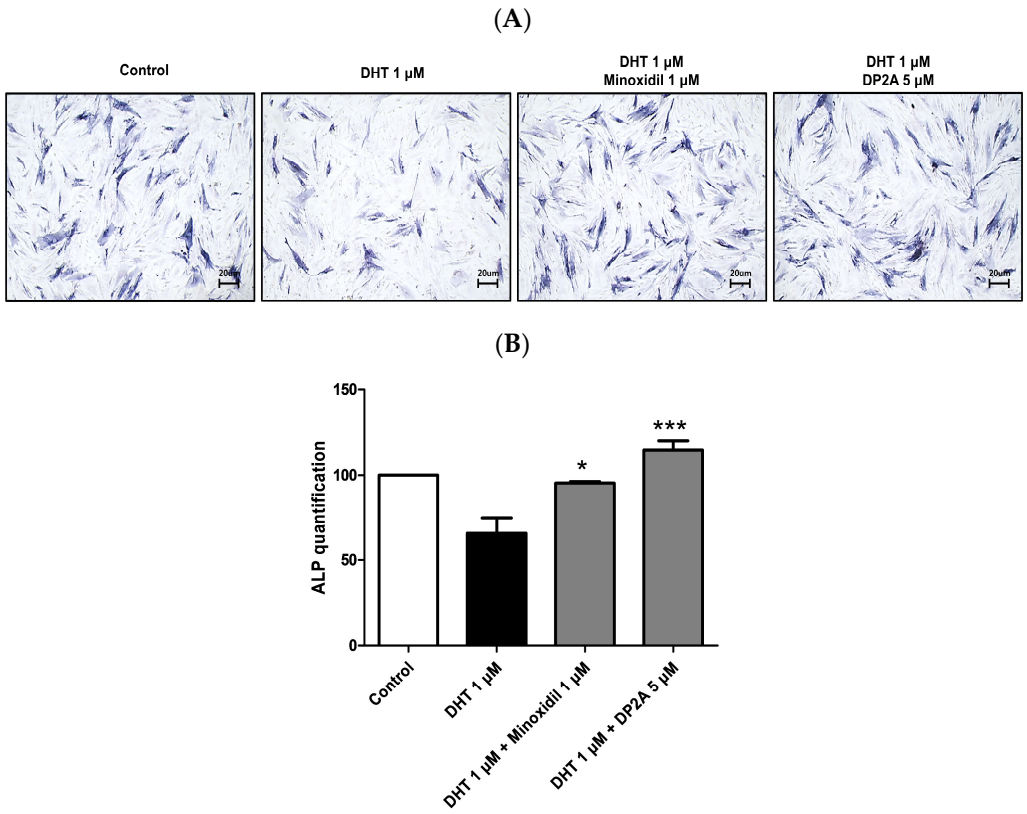


Figure 3. The effects of the DP2A on the ALP expression level in the HFDPCs stimulated with DHT. The ALP assay was performed on the HFDPCs. Cells were each administered with 1 μM DHT, 5 μM DP2A, and 1 μM minoxidil for 24 h. (**A**) This image represents one of three independent experiments. (**B**) The graph was obtained using ImageJ. The data are shown as mean ± SD (n = 3). The significance of the statistics was shown as * $p < 0.05$ and *** $p < 0.001$, compared to the only DHT-treated group.

3.4. The DP2A Decreased the ROS Level in the HFDPCs Damaged by DHT

ROS are generated within cells in response to intrinsic stimuli or extrinsic factors such as particulate matter and ultraviolet light. Excessive ROS cause cellular damage and various diseases [17]. When HFDPCs are exposed to ROS, they can undergo severe damage to the hair follicles [18,19]. These processes interfere with hair growth and maintenance, resulting in hair loss [20]. Minoxidil significantly enhances cell proliferation and reduces intracellular ROS levels in HFDPCs [21]. Therefore, a DCF-DA assay was performed to evaluate the impact of the DP2A on the ROS levels in the HFDPCs that were stimulated with DHT. As expected, the DHT treatment led to an increase in the ROS levels. However,

5 μM DP2A significantly reduced the intracellular ROS levels in the DHT-damaged HFDPCs, bringing them to levels that were comparable to the control (Figure 4).

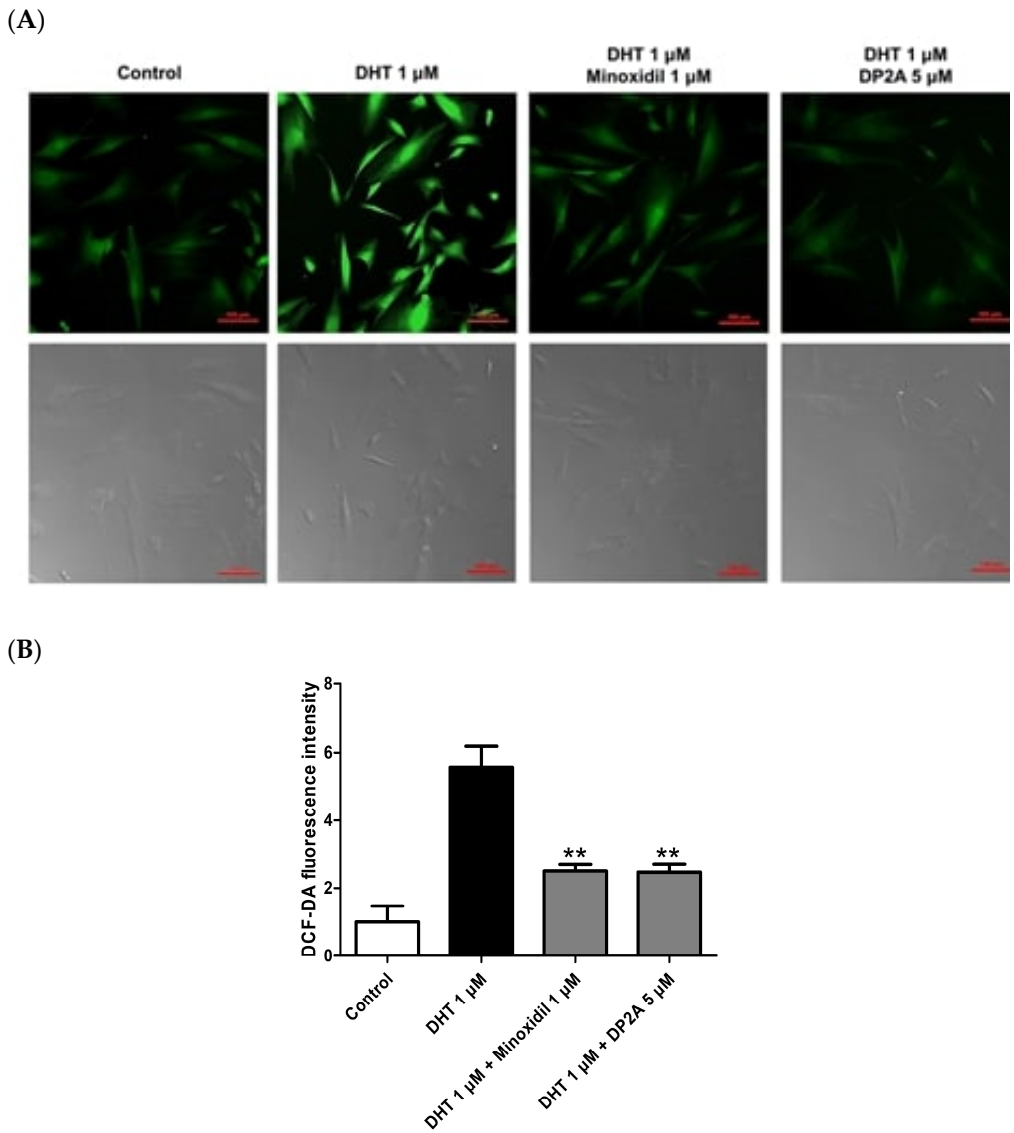


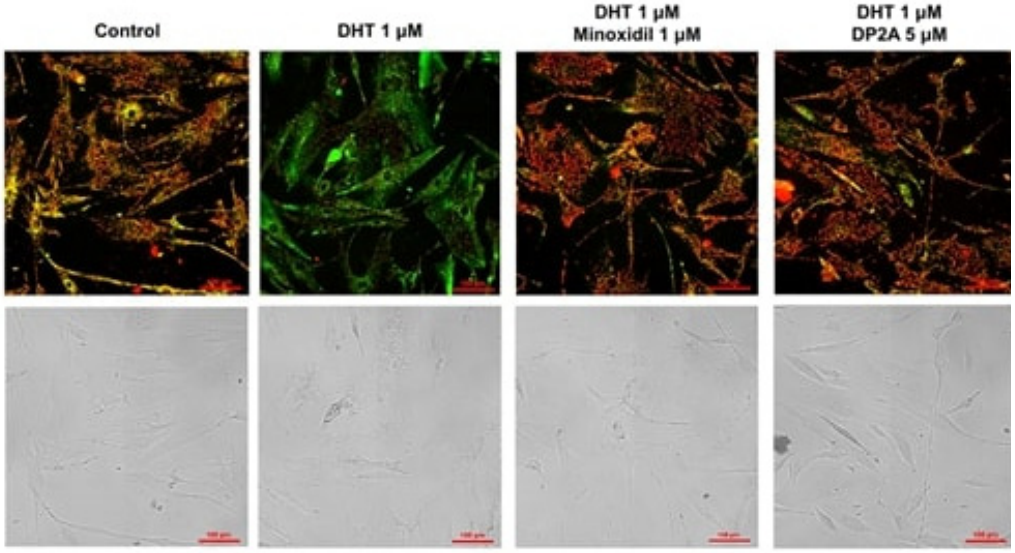
Figure 4. Effects of the DP2A on cellular ROS levels in the HFDPCs stimulated with DHT. The DCF-DA ROS assay was conducted on the HFDPCs. The cells were pretreated with 5 μM DP2A and 1 μM minoxidil for 24 h. Each sample was administered with 1 μM DHT. (**A**) Each image of DCF-DA was obtained using a Nikon Eclipse Ti2 fluorescence live-cell imaging microscope. The green fluorescence intensity means the ROS level. Each image was obtained from one of three independent experiments. (**B**) A graph of the results was obtained using ImageJ. The data are denoted as mean ± SD (n = 3). The significance of statistics was indicated as ** $p < 0.01$, compared to the only DHT-treated group.

3.5. The DP2A Restored the Membrane Potential of Mitochondria in the HFDPCs Damaged by DHT

Mitochondria are key organelles for cellular energy production, and their dysfunction is associated with cellular damage [22]. During oxidative phosphorylation, maintaining the membrane potential of mitochondria (ΔΨm), facilitated by complexes I, III, and IV, is important for energy storage [23]. DHT impairs mitochondrial function and causes cellular damage [24]. We performed a JC-1 assay to examine the membrane potential of mitochondria in the DHT-damaged HFDPCs that were treated with the DP2A. Green fluorescence signifies abnormal potential, whereas red fluorescence means normal mitochondrial membrane potential. As expected, the DHT treatment resulted in increased green fluorescence compared to the control. However, treatment with 5 μM DP2A increased the

red fluorescence in the HFDPCs stimulated with DHT, indicating that the DP2A restored the mitochondrial potential (Figure 5).

(**A**)



(**B**)

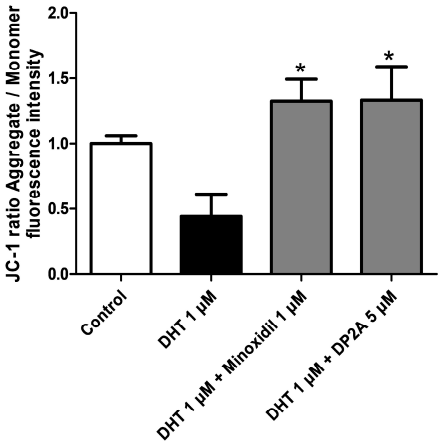


Figure 5. The effects of the DP2A on the membrane potential of the mitochondria in the HFDPCs stimulated with DHT. The JC-1 assay was performed on the HFDPCs. Cells were treated with 1 μM DHT. Cells were then treated with 5 μM DP2A and 1 μM minoxidil for 24 h. (**A**) The image of JC-1 was obtained from a Nikon Eclipse Ti2 fluorescence live-cell imaging microscope. Green puncta indicate mitochondria undergoing depolarization, whereas red puncta indicate mitochondria undergoing hyperpolarization. Each image was obtained from one of three independent experiments. (**B**) A graph was obtained by ImageJ. The data are denoted as mean ± SD (n = 3). A statistical significance was shown as * $p < 0.05$, compared to the only DHT-treated group.

3.6. The DP2A Upregulated the Phosphorylation Levels of ERK and AKT and the Expression Level of β-Catenin in the HFDPCs Damaged with DHT

β-catenin interacts with GSK3β, CK1, APC, and Axin to form a complex in the cytoplasm. When Wnt ligands are active, they bind to their receptors, which prompts the translocation of β-catenin into the nucleus. This translocation then generates the expression of genes related to hair proliferation [25]. The HFDPCs increased their levels of activated ERK and Akt proteins, along with β-catenin [26]. To examine the molecular mechanisms of the DP2A, we performed a western blot assay to measure the phosphorylation levels of ERK and Akt and the expression level of β-catenin.

The DHT treatment decreased the phosphorylation levels of Akt and ERK. It also reduced β-catenin expression. In contrast, treatment with 5 μM DP2A increased the phosphorylation levels of Akt and ERK. It also elevated β-catenin expression (Figure 6). These findings suggest that the DP2A improved hair loss in the HFDPCs stimulated with DHT by activating the Akt/ERK and Wnt signaling pathways.

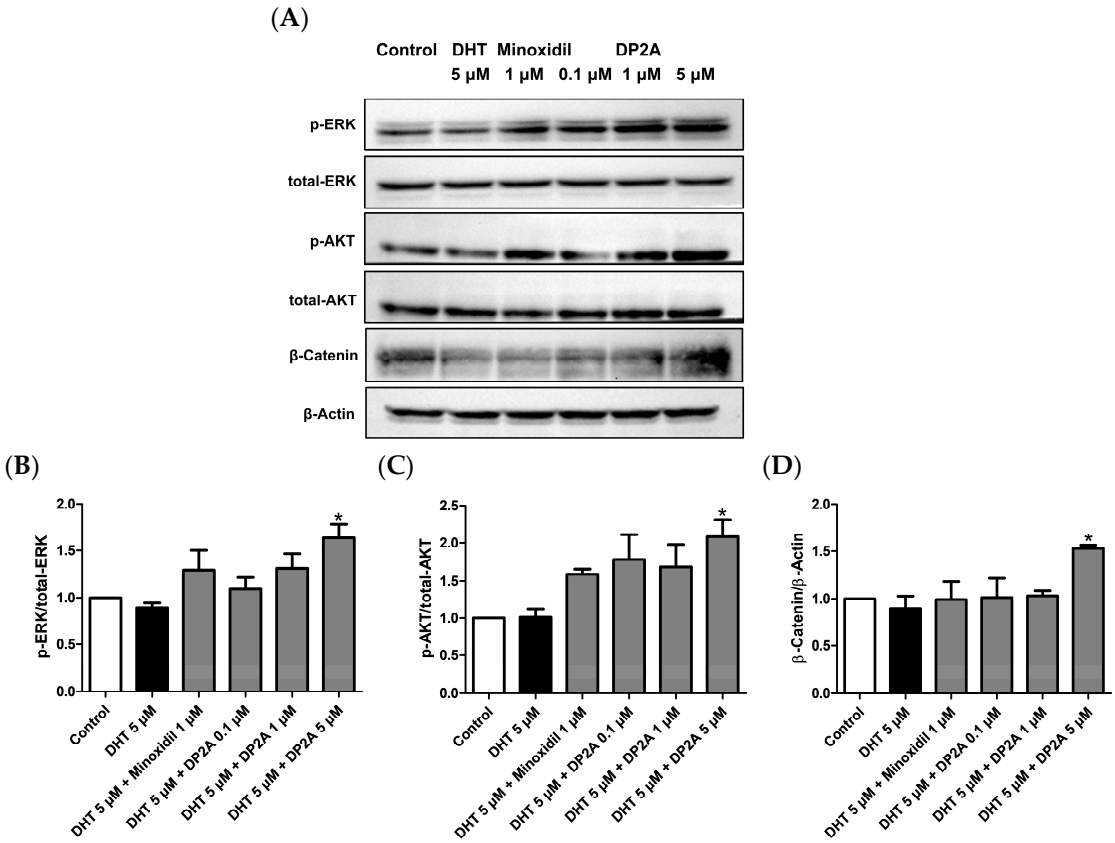


Figure 6. The effects of the DP2A on the phosphorylation level of ERK and AKT and on the β-catenin expression level in the HFDPCs stimulated with DHT. (**A**) Relative band image profile of each protein. (**B**) ERK, (**C**) AKT, and (**D**) β-catenin. Each sample was administered with 5 μM DHT, 0.1, 1, or 5 μM DP2A, and 1 μM minoxidil for 24 h, and then was analyzed using western blotting. Each image represents one of three independent experiments. The data are denoted as mean ± SD (n = 3). A statistical significance was denoated as * $p < 0.05$, compared to the only DHT-treated group.

3.7. The DP2A Enhanced the 3D Spheroid Size in the HFDPCs Damaged with DHT

Three-dimensional spheroids provide an environment closer to that of hair follicles compared to two-dimensional cell cultures, enabling a better understanding of cellular interactions and tissue formation [27]. HFDPCs show stem cell-like self-renewal properties and can proliferate in three-dimensional cultures [28]. A previous study showed that DHT suppresses cell growth [29].

To evaluate the effects of the DP2A on the cell growth of the HDFPCs, we analyzed the formation of 3D spheroids. As expected, the size of the spheroid was diminished in the presence of DHT compared to the control. In contrast, the 5 μM DP2A treatment led to an increase in the spheroid size relative to the DHT-damaged HFDPCs, highlighting the beneficial effect of the DP2A on the HFDPCs (Figure 7).

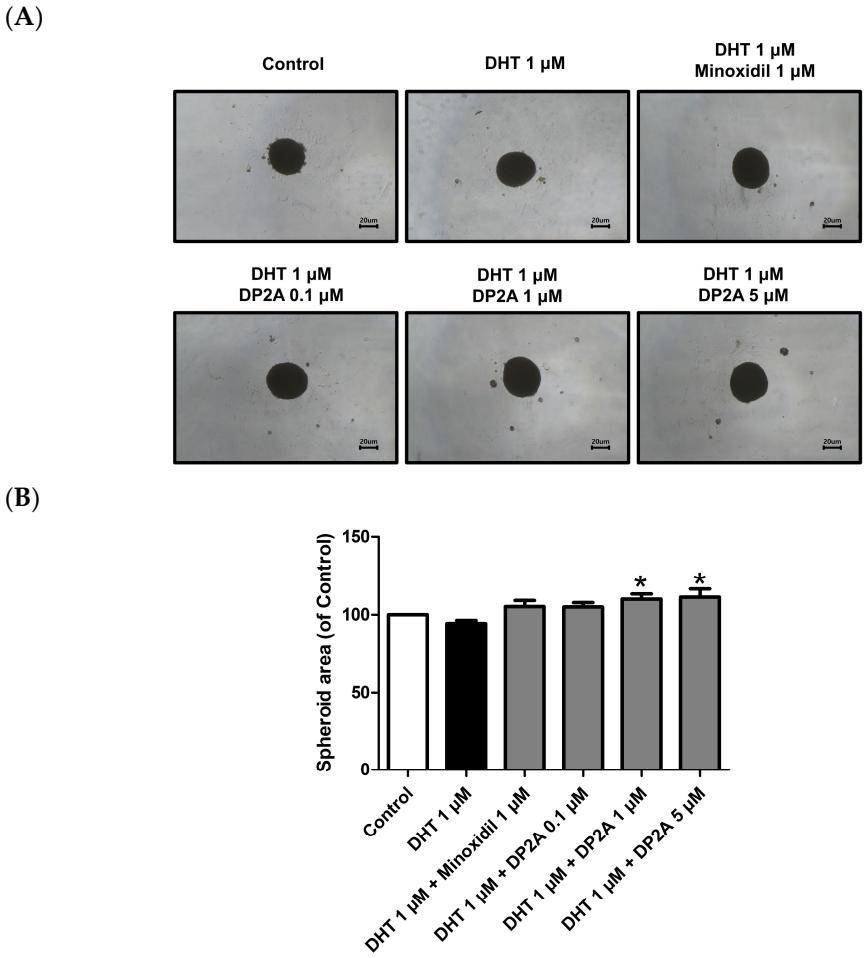


Figure 7. The effects of DP2A on the 3D spheroid formation in the HFDPCs stimulated by DHT. Each sample was administered with 1 μM DHT, 0.1, 1, or 5 μM DP2A, and 1 μM minoxidil at 2-day intervals. The images were obtained after 21 days of culture. (**A**) Each 3D spheroid image was acquired using a microscope. Each image was obtained from one of three independent experiments. (**B**) A graph of the images was obtained by ImageJ. The data are shown as mean ± SD (n = 3). The significance of statistics is indicated as * $p < 0.05$, compared to the only DHT-treated group.

3.8. The DP2A Increased Ex Vivo Hair Growth

Hair follicle organ cultures provide an effective means for studying the associations among cells, such as epithelial, mesenchymal, and neuroectodermal cells. In addition, this model allows for measuring how different natural and pharmacological chemicals affect hair growth [30,31]. To assess the effects of the DP2A on hair growth, we performed a human hair follicle organ culture ex vivo. The minoxidil significantly promoted hair growth compared to the control. Notably, the DP2A also substantially enhanced hair growth relative to the control. On day 8 of the culture, the DP2A increased the hair shaft length by 14.2% (1 μM) and 15.5% (2.5 μM) compared to the control (Figure 8).

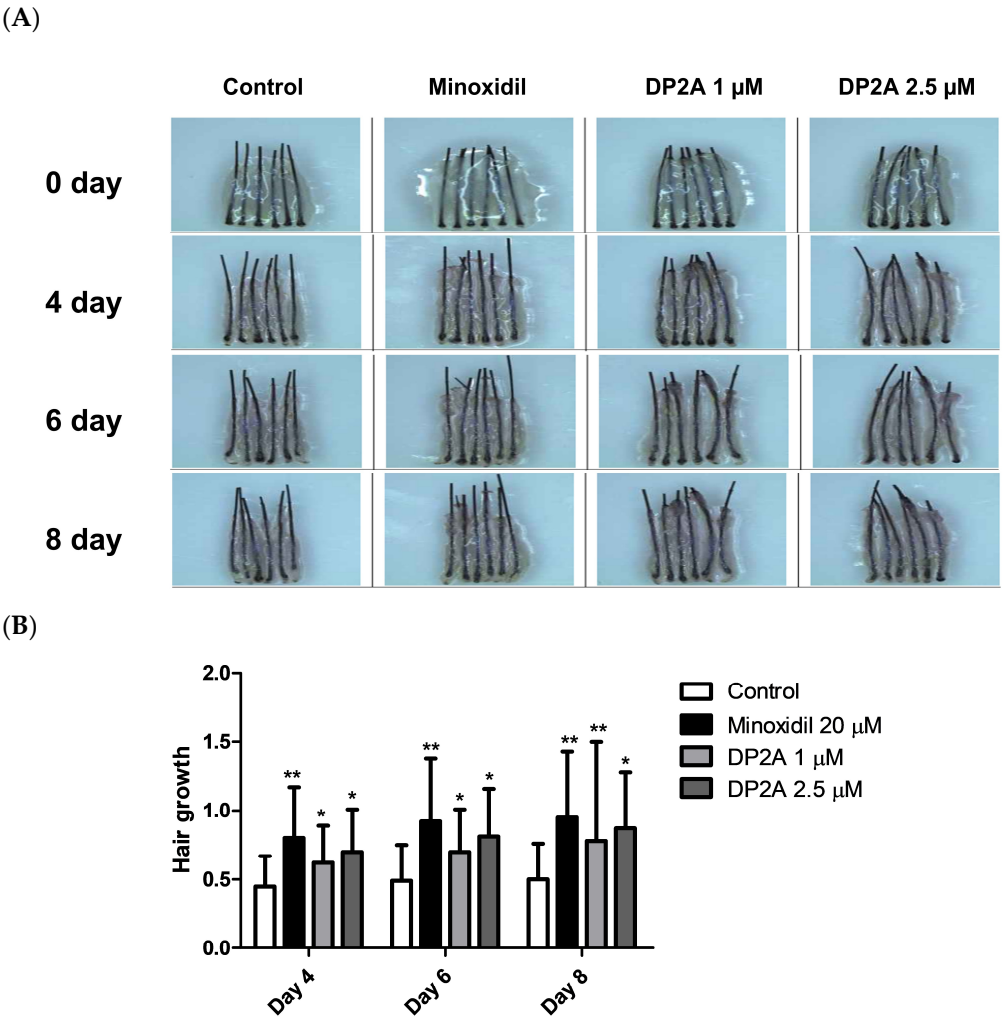


Figure 8. The effects of the DP2A on human hair follicle organ cultures. The anagen status of human hair follicles was chosen to analyze the effect of the DP2A. Each hair follicle organ was cultured for 8 days with DP2A, administered at 1 and 2.5 μM. (**A**) Photographs of the hair follicles were obtained at 4, 6, and 8 days. (**B**) The growth of the hair shaft was analyzed. The data are presented as mean ± SD (eighteen follicles). The *p*-values were acquired by the Mann–Whitney U test. The significance of statistics was denoted as * $p < 0.05$ and ** $p < 0.01$, compared to the control.

4. Discussion

The market for hair loss solutions is growing as the demand for hair loss treatments rises in patients. AGA is primarily caused by dihydrotestosterone (DHT), a derivative of testosterone [32]. AGA causes psychological and social issues due to its impact on hair loss, which results from a shortened anagen phase and an extended telogen phase. Given the side effects involved with minoxidil and finasteride [33], there is a need to develop safer medications for improving hair loss.

Prostaglandins are crucial in the regulation of hair growth and differentiation [34]. Several studies reported that PGD_2 stimulates hair loss [35–37]. Thus, we screened various antagonists of DP2, which targets the PGD2 receptor using DeepZema®, an AI-driven drug development program, and finally identified the DP2A. We examined whether the DP2A could enhance hair growth in the HFDPCs that were stimulated with DHT. The DP2A promoted the migration of cells in the HFDPCs that were damaged with DHT (Figure 2). In addition, it increased the expression level of alkaline phosphatase (Figure 3). ROS are widely recognized as a significant contributor to hair loss [38,39]. Our findings showed that the DP2A significantly reduced ROS levels compared to the group that was

damaged with only DHT treatment (Figure 4). Mitochondrial dysfunction disrupts energy balance, leading to increased ROS production [40]. Notably, the DP2A also restored the mitochondrial membrane potential to be similar to the control group (Figure 5). The 3D spheroid model simulates cell–cell interactions within dermal papilla cells displaying stem cell-like properties [41,42]. This model is crucial for replicating cellular interactions, making it essential for researching hair follicle formation and regeneration. Notably, we found that the DP2A increased spheroid size, indicating its potential to affect hair growth in a follicle-like status (Figure 7). Furthermore, we demonstrated that the DP2A promoted hair growth in ex vivo hair follicle organ cultures (Figure 8).

The initiation of the Wnt signaling pathway induces the expression levels of cell growth genes, facilitating the regeneration and formation of hair follicles [43]. β-catenin associates with APC, GSK3β, Axin, and CK1 in the cytoplasm. When Wnt ligands bind to the frizzled receptor, β-catenin is translocated to the nucleus. It initiates the expression levels of hair growth-related genes. We demonstrated that the DP2A treatment increased β-catenin expression. The DP2A promotes cell growth by preventing the degradation of β-catenin through the AKT and ERK pathways (Figure 6).

In conclusion, we propose that the DP2A could be a promising candidate for relieving PGD_2-induced hair loss. In addition, we expect that the DP2A has advantages in scalp penetration as a cosmetic formulation, since the DP2A is a low-molecular-weight substance.

Author Contributions: Conceptualization, H.W.L. and D.W.S.; formal analysis, H.W.L., H.J., C.Y.J., Y.L., M.K., J.U.S., J.K. and D.C.L.; funding acquisition, D.W.S.; investigation, H.W.L., C.Y.J., H.J., M.K., J.U.S., J.K., S.K. and D.C.L.; methodology, H.W.L.; writing—original draft, D.W.S.; writing—review & editing, S.L., H.D.P., B.C.P. and D.W.S. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Human scalp skin was collected from the patients' non-balding regions. This clinical study was performed with written consent and approval from the Institutional Dankook University Hospital's Review Board (IRB no. DKUH. 2021-12-005).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patients to publish this paper.

Data Availability Statement: The data from this study are available upon demand from the corresponding author.

Conflicts of Interest: There are no conflicts of interest to disclose. This collaboration with Innovo Therapeutics Inc., Hyunwoo Joo, Yurim.Lee, Sanghwa Lee, Dong Chul Lim, and Hee Dong Park, is grounded in a mutual research and development framework. This partnership utilized laboratory facilities and advanced pharmaceutical technologies. Although Innovo Therapeutics Inc. contributed chemical support, it did not influence the research outcomes. The results reflect objective scientific investigation and academic independence.

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