BIOMOLECULES & THERAPEUTICS

Hair Growth Promotion by δ-Opioid Receptor Activation

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Abstract

Literature has revealed that the delta opioid receptor (DOR) exhibited diverse pharmacological effects on neuron and skin. In the present study, we have investigated whether the activation of DOR has hair-growth promotion effects. Compared with other opioid receptor, DOR was highly expressed in epidermal component of hair follicle in human and rodents. The expression of DOR was high in the anagen phase, but it was low in the catagen and telogen phases during mouse hair cycle. Topical application of UFP-512, a specific DOR agonist, significantly accelerated the induction of the anagen in C_3H mice. Topical application of UFP-512 also increased the hair length in hair organ cultures and promoted the proliferation and the migration of outer root sheath (ORS) cells. Similarly, pharmacological inhibition of DOR by naltrindole significantly inhibited the anagen transition process and decreased hair length in hair organ cultures. Thus, we further examined whether Wnt/β-catenin pathway was related to the effects of DOR on hair growth. We found that Wnt/β-catenin pathway was activated by UFP-512 and siRNA for β-catenin attenuated the UFP-512 induced proliferation and migration of ORS cells. Collectively, result established that DOR was involved in hair cycle regulation, and that DOR agonists such as UFP-512 should be developed for novel hair-loss treatment.

Key Words: δ-opioid receptor, UFP-512, Outer root sheath cells, Hair growth, Anagen, Wnt/β-catenin

INTRODUCTION

UFP-512, an agonist of the delta opioid receptor (DOR), is known to exhibit diverse pharmacological effects on neuron (He *et al*., 2013; Sheng *et al*., 2018). UFP-512 was initially developed for treatment of mood disorder (Aguila *et al*., 2007) and shown to elicit robust anxiolytic- and antidepressant-like effects in rodents (Vergura *et al*., 2008). Subsequent studies have demonstrated other beneficial effects of UFP-512. For example, UFP-512 activated the motor activity in hemiparkinsonian rats by controlling the nigro-thalamic pathway (Mabrouk *et al*., 2009). The activation of the DOR by UFP-512 induced the protection of cortical cells against hypoxic/ischemic insults (Yang *et al*., 2012) and protected cells against oxidative damages by inducing Nrf2 (Cao *et al*., 2015). The activation of DOR also inhibited the production of inflammatory cytokines during neonatal hypoxic-ischemic encephalopathy (Qiu *et al*., 2019). However, whether UFP-512 might possess the hair growth-promoting effect is unknown at present.

There exist three types of opioid receptor: the mu (μ) opioid receptor (MOR), the delta (δ) opioid receptor (DOR), and the kappa (κ) opioid receptor (KOR). These opioid receptors are highly homologous in protein sequence and belong to the family of G protein-coupled receptors (GPCRs) (Zaki *et al*., 1996). Although the expression and the function of opioid receptors are poorly characterized in the skin and the hair, the role of opioids in protecting the skin and regenerating the hair growth was previously recognized. For example, opioids accelerated the wound healing by upregulating the expression of endothelial and inducible nitric oxide synthase and the vascular endothelial-derived growth factor receptor, Flk1 (Poonawala *et al*., 2005). Morphine also stimulated the migration of oral epithelial cells via the activation of DOR (Charbaji *et al*., 2012). Consistent with these observations, the genetic deletion of DOR altered skin differentiation and delayed wound healing (Bigliardi-Qi *et al*., 2006), and the activation of the DOR promoted cutaneous wound healing by promoting intercellular adhesion and migration in keratinocytes (Bigliardi *et al*., 2015). β-endorphin also stimulated the expression of cytokeratin 16 and downregulated the expression of MOR in

human epidermis (Bigliardi-Qi *et al*., 2000). Notably, topical treatment of the opioid antagonist, low-dose naltrexone (LDN), facilitated the closure of full-thickness wounds in diabetic rats (McLaughlin *et al*., 2011): LDN is a competitive antagonist of μ, κ and γ opioid receptors, and is used for treating alcoholism and opioid addiction. Exposure of LDN at low dosages exhibited a paradoxical effect by increasing the production of endogenous opioids, including β-endorphins, exhibiting anti-inflammatory properties. Therefore, it was proposed that LDN can be an adjunctive treatment for symptomatic alopecia at appropriate dosages (Tortelly *et al*., 2019).

It is expected that opioid receptors are expressed in epidermal cells, promoting skin regeneration (Neumann *et al*., 2015; Bigliardi *et al*., 2016), but the effects of opioid receptors on hair regeneration are unclear. Therefore, we have investigated which types of opioid receptors are expressed in hair follicles and, if so, whether they could promote the hair growth together with the underlying molecular mechanisms.

MATERIALS AND METHODS

Cell culture

Human follicle ORS cells, isolated from hair follicles of human scalp skin were cultured in EpiLife™ Medium with 60 μM calcium chloride, 1% EpiLife™ Defined Growth Supplement (EDGS) (Thermo Fisher Scientific, Waltham, MA, USA), and 1% antibiotic-antimycotics (Thermo Fisher Scientific). ORS cells were maintained in a humidified incubator at 37°C under 5% CO_2.

Cell proliferation assay

Human ORS cells (passage 3) were plated in 48-well plates in triplicates at a density of 6,000-7,000 cells per well. After 24 h, cell culture medium was replaced with serum-free basal medium containing 1% anti-antibiotics and exposed to UFP-512 for 48 h. In order to measure the rate of cell proliferation, the medium was replaced with prepared CCK-8 solution (Dojindo Molecular Technologies, Rockville, MD, USA), then incubated at 37°C under 5% CO_2 for 2 h and the absorbance was measured at 450 nm using a microplate reader (Tecan, Männedorf, Switzerland).

Wound healing assay

The wound healing assay was conducted to determine the migration of ORS cells. In brief, ORS cells (passage 3; 5×10^5 cells/ well) were seeded in 35 mm culture dishes overnight. Confluent ORS cells were wounded using a sterile blue pipette tip, washed with DPBS to remove detached cells, and exposed to vehicle or UFP-512 for 48 h. Cell migration was determined via microscopic examination at five points randomly selected and the horizontal distances of cell migration from the edge of wound were measured.

Real-time quantitative reverse transcription-polymerase chain reaction (real-time quantitative RT-PCR)

Total RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific), followed by reverse transcription using a cDNA synthesis kit (Nanohelix, Daejeon, Korea). Real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed using the StepOne Real-Time PCR System (Thermo Fisher Scientific). GAPDH was used as an internal control to normalize the changes in gene expressions.

Human WNT signaling pathway polymerase chain reaction array

Gene expression profiles of ORS related with the WNT signaling pathway was analyzed using the Human Transduction Pathway Finder RT² Profiler PCR Array (PAHS-043ZA, Qiagen, Hilden, Germany). Fold changes in expression were calculated using the ΔCt value. Data analysis is based on the $^{\Delta\Delta}CT$ method after normalization of the raw data to housekeeping genes. The red lines indicate ± 2 folds change in gene expression threshold. The X axis indicates the gene expression changes after vehicle treatment and the Y axis indicates the gene expression changes after treatment of 1 μM UFP-512.

Western blotting

In order to prepare whole cell lysates, adherent ORS cells (passage 3) were washed by PBS and immersed in protein extraction solution (Intron, Seoul, Korea) after scraping. Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% gels and transferred to PVDF membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% skim milk in TBST (0.1% Tween-20 in Tris-buffered saline) for 1 h at room temperature and incubated overnight with primary antibody at 4°C. At the next day, the membranes were washed three times with TBST and incubated with HRP (horseradish peroxidase)-conjugated secondary antibodies for 1 h at room temperature. The band image of the membrane was acquired by using enhanced chemiluminescence solution (Millipore). Western blotting of β-actin was conducting to visualize the equal sample loading. The list of primary antibodies used in the present study are illustrated in Table 1.

The induction of anagen in mice

Mice were maintained and anesthetized according to a protocol approved by Kim *et al* (2015). This experiment was ap-

Table 1. Primary antibodies used for immunofluorescence analysis and western blot

Antibodies	Species	Source	Dilution (WB)	Used to identify
ERK	Rabbit	Cell Signaling Technology (Danvers, MA, USA)	1:1,000	ORS cell
p-ERK	Mouse	Cell Signaling Technology	1:1,000	ORS cell
p-AKT	Rabbit	Cell Signaling Technology	1:1,000	ORS cell
^{S552}β-catenin	Rabbit	Cell Signaling Technology	1:1,000	ORS cell
β-catenin	Rabbit	Cell Signaling Technology	1:1,000	ORS cell
β-actin	Mouse	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	1:2,000	ORS cell

proved by the Institutional Animal Care and Use Committee of Yonsei University (Seoul, Korea) (IACUC120002). In brief, the dorsal area (2.5 cm×4 cm) of 7 week-old C_3H/HeN male mice in the telogen stage of the hair cycle was shaved using a clipper and electric shaver with a special care taken to avoid the damages in the bare skin. Control or 0.01-0.05% UFP-512 was topically applied onto the back of mice for 2 weeks and any darkening of the skin, e.g. indicative marker for hair cycle induction, was carefully monitored by photography. At 15th day post-experiment, dorsal hair was shaved and its weight was measured. The number of anagen hair shafts was counted after haematoxylin and eosin (H&E) staining. UFP-512 was provided by our team members, Prof. Gianfranco Balboni and Prof. Ying Xia.

Skin biopsies

For depilation experiments, 7-week-old male mice were anesthetized with isoflurane and hairs in a 2.5 cm×4 cm area of mid-dorsal skin were manually plucked with wax strips to induce synchronized hair cycling. Hair follicles of mice entering the telogen phase were confirmed by pink to white color change of the skin. Dorsal skin biopsies were taken from euthanized mice by CO_2 inhalation. Hairs on the back of mice were carefully shaved using an electric clipper before harvesting skin biopsies. Collected skin tissues were processed for preparation of paraffin block.

Primary hair organ culture

The hair growth promoting activity of mouse vibrissae was observed by using primary hair organ culture. Hair follicles (HFs) were isolated and cultured according to the method previously described by Jindo and Tsuboi (Jindo *et al.*, 1994). Normal vibrissal HFs in the anagen phase were obtained from the upper lip region of mice using a scalpel and tweezers. Isolated murine vibrissal HFs were placed in a defined medium (serum-free Williams E medium supplemented with 2 mM L-glutamine, 10 μg/mL insulin, 10 ng/mL hydrocortisone, 100 U/mL penicillin, and 100 μg/mL streptomycin) and exposed to vehicle or UFP-512 at various concentrations. Individual HFs were photographed 72 h after the start of incubation, using (Edmund Optics Ltd, UK) and the changes in hair length were calculated based on the images. Values are expressed as mean ± standard error (SE) of 10-12 vibrissal HFs.

Table 2. The human specific primer sequences

β-catenin	Forward primer	5`-CGUUCUCCUCAGAUGGUGU-3`
	Reverse primer	5`-ACACCAUCUGAGGAGAACG-3`
DOR	Forward primer	5`-CCAAGGCCAAGCUGAUCAA-3`
	Reverse primer	5`-UUGAUCAGCUUGGCCUUGG-3`

Small interfering RNA (siRNA) transfection

Cells were seeded in 35 mm culture dish at a density of $5×10^4$ cells/well and allowed to reach approximately 60% confluence on the day of transfection. siRNA constructs were ordered from Bioneer corporation (Daejeon, Korea) and their sequences are illustrated in Table 2. Cells were transfected with 20 nM siRNA using Lipofectamine™ RNAiMAX Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions.

Immunofluorescence staining *in vitro*

Human ORS cells were fixed with formaldehyde. After washing with 1x PBS, cells were exposed to primary antibodies overnight at 4°C. Cells were washed three times with 1x PBS and stained with Alexa Fluor 488-goat anti-rabbit IgG for 1 h or with 4,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA) for visualizing the nucleus. The immunofluorescent images was acquired using a Zeiss LSM700 confocal microscope (Ca Zeiss, Oberkochen, Germany). Primary antibodies used in this study are presented in Table 3.

Immunofluorescence staining *in vivo*

Human HFs were kindly received from Prof. Sang Ho Oh (Severance Hospital, Yonsei, University College of Medicine, Seoul, Korea). Unstained paraffin slides were deparaffinized and the antigen retrieval was performed using citrate buffer (Scy Tek, Logan, UT, USA). Slides were then blocked with 1% bovine serum albumin (BSA) in 1x PBS for 1 h at RT before the application of primary antibodies overnight at 4°C. After the slides were washed with 0.05% Triton-PBS for 3 times, the slides stained with Alexa Fluor 488-goat anti-rabbit IgG or Alexa Fluor-594 goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) for 1 h at RT with DAPI. The immunofluorescent images were acquired using a Zeiss LSM700 confocal microscope (Ca Zeiss). Primary antibodies used in this study are presented in Table 3.

Statistical analysis

All data are expressed as the mean ± standard deviation (SD) of three independent experiments. Statistical differences were evaluated using one-way ANOVA or Student's t-test.

RESULTS

Delta opioid receptor (DOR) is dominantly expressed in hair follicles

We first examined the expression levels of DOR, MOR, and KOR in human and mouse hair fibroblasts (HFs) by immunofluorescence staining. As a result, we observed that DOR, but not MOR and KOR, was selectively expressed in the ORS
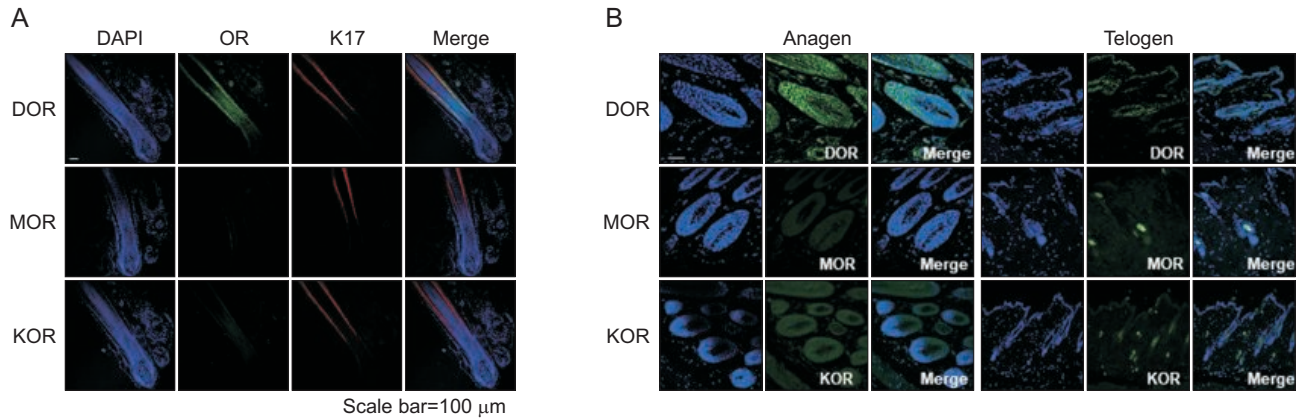
Table 3. Primary antibodies used for immunofluorescence staining *in vivo*

Antibodies	Species	Source	Dilution (IF)	Used to identify
DOR	Rabbit	Abcam (Cambridge, MA, USA)	1:200	Hair follicle & ORS cell
MOR	Rabbit	Invitrogen	1:200	Hair follicle
KOR	Rabbit	Prosci (San Diego, CA , USA)	1:200	Hair follicle
S363DOR	Rabbit	Biorbyt (Cambridge, GB, UK)	1:200	Hair follicle & ORS cell
Keratin 17	Mouse	Santa cruz	1:100	Hair follicle

Fig. 1. Delta opioid receptor expression in the hair follicle. (A) Immunostaining showed that among the 3 opioid receptors, only DOR (green) highly expressed and localized in the ORS region of HFs and co-localized with keration 17 (k12, red, an ORS marker) in the HF. (B) DOR was detected in the HFs in the anagen phase, and weakly expressed in the hair germ and bulge area during telogen phase. MOR and KOR were difficultly detected in the HFs in both anagen and telogen phase. DAPI staining (blue) indicates nuclei.
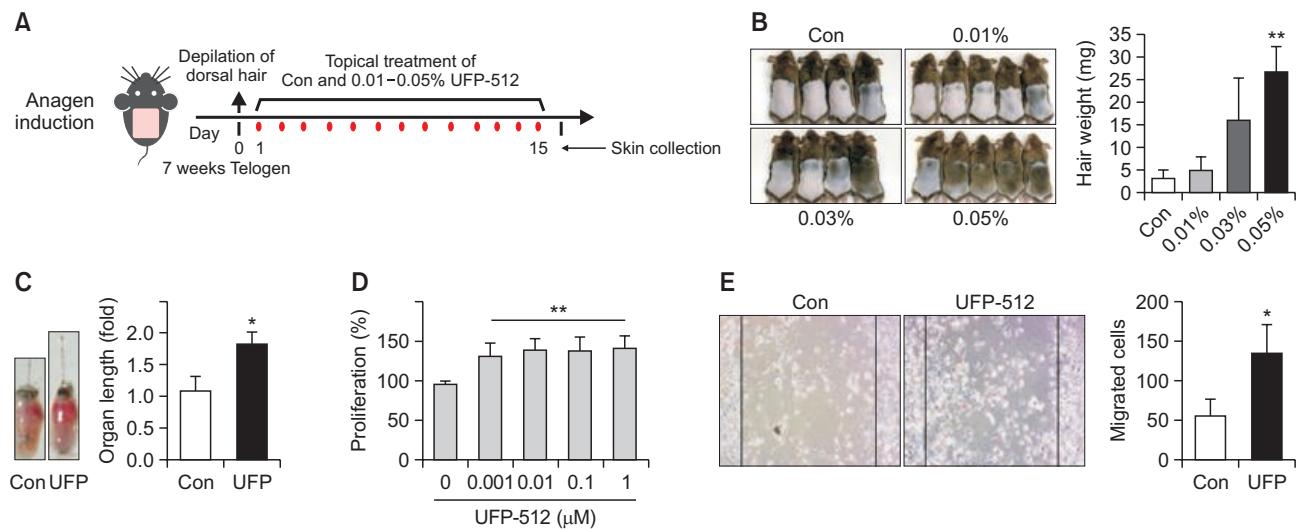

Fig. 2. Hair growth-promoting effects of UFP-512. (A) The experiment design. The back skin of 7-week-old C_3H mice was shaved, and 0.01%-0.05% UFP-512 was topically applied. (B) Compared with the negative control, topical application of UFP-512 facilitated the telogen-to-anagen transition in C3H mice at day 15. Dorsal hair weight was measured. $**p<0.01$. (C) UFP-512 treatment promoted mouse vibrissa follicle growth *ex vivo*. $*p<0.05$, n=10 mouse vibrissae follicle per group. The proliferation (D) and migration (E) of hORS cells was measured. $*p<0.05$, $**p<0.01$.

region of human scalp HFs: keratin 17 (k17) was costained as ORS marker 17 (Fig. 1A). Similarly, DOR was selectively expressed in ORS cells of mice HFs during the anagen phase and its level decreased during the telogen phases (Fig. 1B). Together, these results suggest that DOR might be associated with the regulation of hair cycling.

UFP-512 induced the growth of mouse hair, and the proliferation and the migration of hORS cells

We used 7 week-old male C_3H/HeN mice in the telogen phase to investigate the telogen-to-anagen transition by UFP-512 (Fig. 2A). As a result, we observed that topical application of UFP-512 (0.01%-0.05%) facilitated the telogen-to-anagen transition in C_3H/HeN mice at day 15 in a dose-dependent manner (Fig. 2B). We also evaluated the hair growth-promot-

ing effect of UFP-512 in murine vibrissa organ cultures and found that UFP-512 increased the growth of hair in murine vibrissae *ex vivo* (Fig. 2C). In addition, we observed that UFP-512 significantly promoted the proliferation (Fig. 2D) and migration of hORS cells (Fig. 2E).

The inhibition of DOR attenuated the hair growth and hORS cell proliferation

Because of a high expression of DOR of HFs in the anagen phase than the telogen phase, it can be assumed that DOR might be associated with the regulation of the hair-growth cycle. Supporting for this notion, we observed that subcutaneous injection of UFP-512 significantly increased DOR phosphorylation of HFs in the anagen phase (Fig. 3A) and cultured hORS cells (Fig. 3B). Next, we used siRNA to decrease the
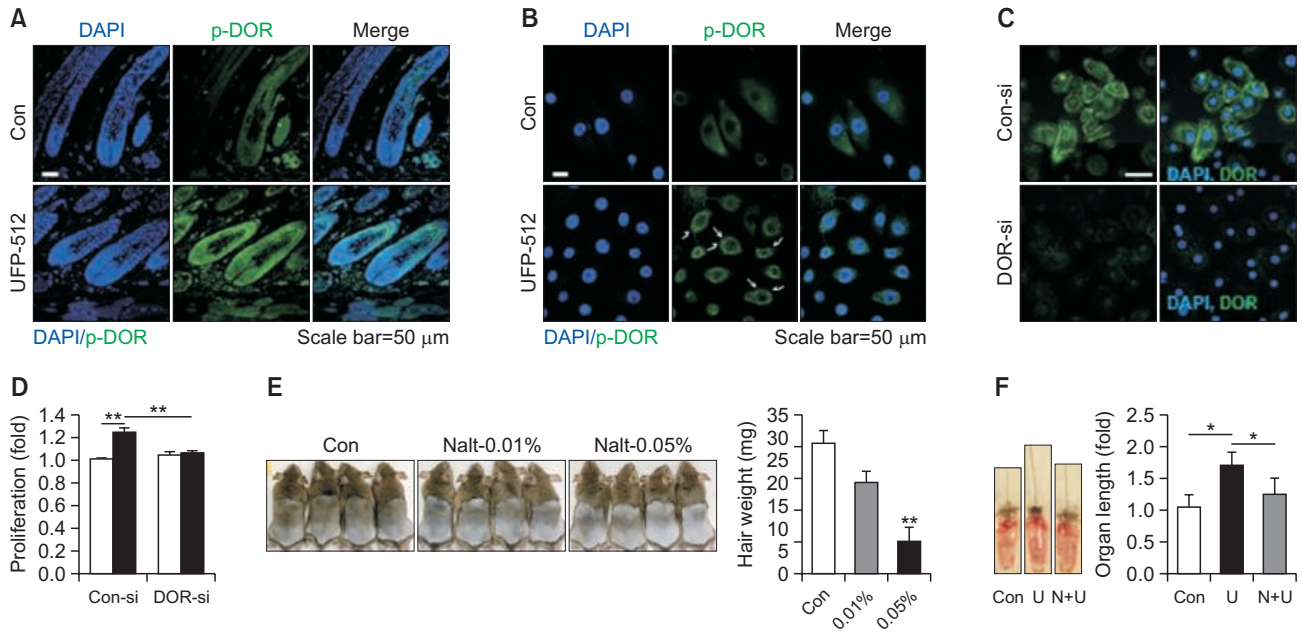
Fig. 3. Activation of DOR in hair follicle and ORS cells. (A) 4-week-old C_3H mice was used. Phosphorylation of DOR was detected at 30 min after 1 μM UFP-512 subcutaneous injection into the dorsal skin of shaved mice. Increased p-DOR was observed in matrix and ORS cells at anagen HFs of UFP-512 treated mice. Scale bar=50 μm. (B) DOR was activated and phosphorylated by 1μM of UFP-512 in hORS cells (as indicated by the arrow in immunostaining). Scale bar=20 μm. DAPI staining (blue) indicates nuclei. (C, D) Proliferation was monitored in con- and DOR-siRNA treated hORS cells. DOR knockdown was achieved using siRNA transfection and immunostaining. **$p<0.01$. (E) Compared with the negative control, topical application of naltrindole (0.01%-0.05%) significantly delay the telogen-to-anagen transition in C_3H mice. Dorsal hair weight was measured. **$p<0.01$. (F) UFP-512 induced hair growth was attenuated by Naltrindole. *$p<0.05$.

expression level of DOR in cultured hORS cells (Fig. 3C), Results also showed a UFP-512-induced increase in the proliferation of ORS cells that was reduced by DOR siRNA (Fig. 3D). In addition, DOR pharmacological inhibition was tested. The specific antagonist, naltrindole, attenuated UFP-512-induced hair growth in mice vibrissa organ culture. Furthermore, 7 week-old male C₃H/HeN mice in the telogen phase was used to investigate the telogen-to-anagen transition by naltrindole. Compared with the negative control, topical application of naltrindole (0.01%, 0.05%) significantly delayed the telogen-to-anagen transition in C₃H/HeN mice (Fig. 3E). Naltrindole significantly inhibited the hair weight in a dose-dependent manner as well (Fig. 3F). These results imply that both protein level and activity of DOR are important for regulating the hair-growth cycle.

UFP-512 regulated Wnt/β-catenin signaling in hORS cells

We next explored the underlying mechanism of hair growth promotion by UFP-512 in cultured hORS cells, as studies have demonstrated that DOR signaling specifically activated the ERK1/2 MAPK pathway in human keratinocyte (Neumann *et al*., 2015). To confirm this fact, the hORS cells were treated with UFP-512 for 0–120 min. UFP-512 increased phosphorylation of AKT and ERK1/2 (Fig. 4A). Furthermore, UFP-512 also increased active β-catenin (ser552) expression, which protects its degradation from GSK3β (Aberle *et al*., 1997).

It has been reported that opioids can activate the canonical Wnt/β-catenin signaling pathway (Li *et al*., 2019). Activation of the Wnt/β-catenin pathway is important for HF regeneration and hair shaft growth (Huelsken *et al*., 2001; Collins *et al*., 2011). Therefore, we hypothesized the Wnt/β-catenin

pathway to be involved in UFP-512 induced hair growth. We measured the mRNA levels of the Wnt pathway-related genes using QPCR array after UFP-512 treatment. UFP-512 up-regulated the mRNA levels of seven genes (WNT1, WNT2, WNT6, WNT16, WNT11, WISP, and AXIN2) (fold change > 2) among the 84 Wnt pathway-related genes in cultured hORS cells (Fig. 4B, 4C). Also, to investigate whether β-catenin is involved in UFP-512-regulated proliferation of ORS, we performed a proliferation assay using siRNA for β-catenin. Results showed that the β-catenin siRNA treatment decreased UFP-512 induced proliferation of hORS cells (Fig. 4D).

DISCUSSION

DOR exhibits diverse pharmacological effects on neuron and skin (Bigliardi-Qi *et al*., 2006). Therefore, we tried to investigate whether DOR activation has hair-growth promotion effects in this study. Compared to other opioid receptors, DOR was highly expressed in epidermal component of hair follicles in humans and rodents. DOR expression was high in anagen and low in catagen and telogen of mice hair-growth cycle. Topical treatment of UFP-512, a specific DOR agonist, significantly accelerated anagen induction in C₃H mice. UFP-512 also increased the hair length in hair organ cultures and enhanced proliferation as well as migration of hORS cells and increased the phosphorylation of DOR in hair follicles and hORS cells as well. Alternatively, pharmacological inhibition of DOR by naltrindole significantly delayed anagen transition and decreased hair length in hair organ culture as well. We also found that Wnt/β-catenin pathway was activated by UFP-
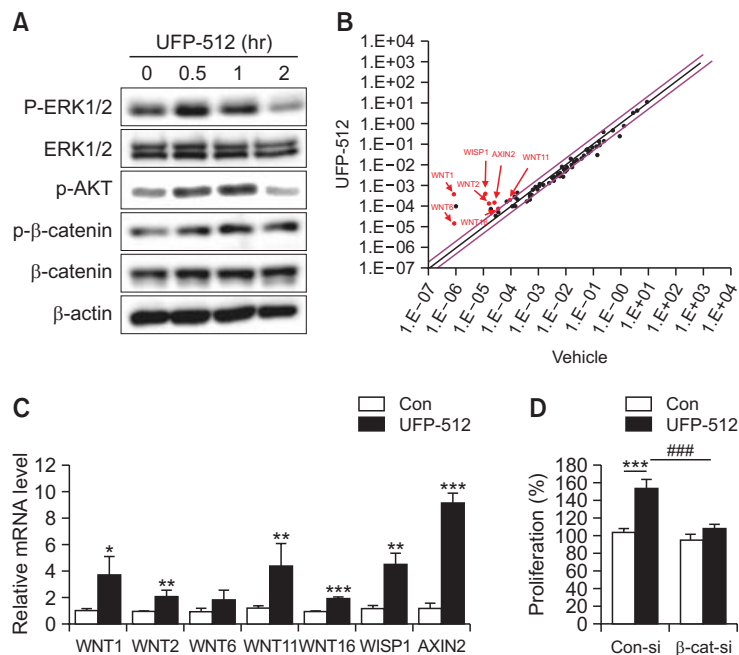
Fig. 4. Involvement of AKT, MAPK/ERK and Wnt/β-catening signaling pathway. (A) AKT and MAPK/ERK signaling pathways were assessed under UFP-512 treatment using western blot. (B) Wnt signaling pathway was observed using a RT^2 profiler PCR assay. Among the 84 Wnt-related genes, seven genes were increased (WNT1, WNT2, WNT6, WNT16, WNT11, WISP, and AXIN2). (C) qRT-PCR was used to confirm upregulation of mRNA expression. *$p<0.05$, **$p<0.01$, ***$p<0.001$. (D) Involvement of β-catenin in UFP-512 induced proliferation of ORS was observed using β-catenin siRNA transfection. ***$p<0.001$ *vs.* cells exposed to con; & $^{###}p<0.001$ *vs.* β-cat-si/UFP-512 treated cell to con-si/UFP-512.

512, and siRNA for β-catenin attenuated the UFP-512 induced proliferation of hORS cells. Collectively, results showed that DOR was involved in hair-growth cycle regulation, and DOR agonists exhibit hair-growth promoting effects.

Literature has shown that β-endorphin, one of the prominent endogenous opioid receptor ligands, was expressed in the skin of C57BL/6 mouse, and accumulated in the pilosebaceous apparatus during the hair-growth cycle (Furkert *et al.*, 1997). They also demonstrated that anagen-associated increase in β-endorphin concentration and its decline during the catagen/telogen phase raise the possibility of a regulatory function of DOR in cyclic changes of hair follicles. However, they did not examine the opioid receptor expression and functional involvement. We first investigated in this study, which receptor was expressed and involved in hair-growth progression. Among the subtype of opioid receptors, only DOR was predominantly expressed in human and mouse hair follicles. We also measured the expression of DOR during the anagen to telogen phase in C$_3$H mice. Interestingly, DOR was highly expressed in ORS and matrix cells during hair-growth period (anagen phase) compared with hair regress period (telogen phase). Collectively, these indicate that DOR plays a key role in hair-growth, and is involved in the regulation of hair-growth cycle (Fig. 1).

UFP-512 was shown to behave *in vitro* as a selective and potent DOR agonist and to promote antidepressant- and anxiolytic-like effects *in vivo* (Vergura *et al.*, 2008). UFP-512 has been demonstrated up to 1000-fold more potent to compete [^{3}H]-diprenorphine for hDOR binding than other DOR selective and nonselective opioid agonists (Polastron *et al.*, 1994; Marie *et al.*, 2003), and displayed a high affinity for the DOR that was associated with a high selectivity over MOR (160-fold) and KOR (3500-fold) sites. We find that UFP-512 induced ORS proliferation, migration, and increased HF length in the mice organ cultures. We also demonstrated for the first time that UFP-512 accelerated the telogen-to-anagen transition in C$_3$H mice (Fig. 2). Phosphorylation of DOR was also increased by subcutaneously injection of UFP-512 in 4-week-old C$_3$H/HeN mice. In addition, blockade of DOR by transfection of DOR-siRNA or treatment with naltrindole (a specific antagonist of DOR) decreased UFP-512-induced hair growth in a telogen-to-anagen transition model. Collectively, these indicate that DOR agonist should be a new target for development as an anti-hair-loss drug.

The Wnt/β-catenin pathway regulates cell proliferation and is important for the hair-growth cycle regulation, hair morphogenesis, and hair regeneration (Andl *et al.*, 2002; Ito *et al.*, 2007). Epidermal Wnt is required for activation of β-catenin signaling pathway, not only within the epidermis, but also across the epidermis to the dermis (Fu and Hsu, 2013). Forced activation of β-catenin signaling caused thickening of dermis and enlargement of placodes and dermal condensates in mice, resulting in the initiation of epidermal HFs (Chen *et al.*, 2012). Of note, various opioids could activate the canonical Wnt/β-catenin signaling pathway (Wang *et al.*, 2017). For example, agonist-dependent MOR activation modulates Wntless activity, a protein that mediates WNT function by regulating its secretion (Reyes *et al.*, 2012). Therefore, we hypothesized that UFP-512 promoted hair growth via the Wnt signaling pathway. As expected, UFP-512 upregulated the mRNA level of WNT1, WNT2, WNT6, WNT11, WNT16, WISP1, and AXIN2, and increased the active form of β-catenin (Ser552). Therefore, it

is reasonable to assume that UFP-512 can stimulate hORS proliferation and promotes hair growth via Wnt/β-catenin signaling pathway activation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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