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KY19382, a novel activator of Wnt/β-catenin signaling, promotes hair re-growth and hair follicle neogenesis

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Data Availability Statement:

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/bph.15438

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Background and Purpose: The promotion of hair regeneration and growth heavily depends on the activation of Wnt/β-catenin signaling in the hair follicle, including dermal papilla (DP). KY19382, one of the newly synthesized analogs of indirubin-3'-monoxime (I3O), was identified as a Wnt/β-catenin signaling activator via inhibition of the interaction between CXXC-type zinc finger protein 5 (CXXC5) and Dishevelled (Dvl) interaction. Given the close relationship between the Wnt/β-catenin signaling and hair regeneration, we investigated the effect of KY19382 on hair re-growth and hair follicle neogenesis.

Experimental Approach: *In vitro* hair induction effects of KY19382 was performed in human dermal papilla cells. The hair elongation effects of KY19382 was confirmed through the human hair follicle and vibrissa culture system. *In vivo* hair regeneration abilities of KY19382 was identified in three models: hair regrowth, wound-induced hair follicle neogenesis (WIHN) and hair patch assays using C57BL/6 mice. The hair regeneration abilities were analyzed by immunoblotting, alkaline phosphatase (ALP) and immunohistochemical staining.

Key results: KY19382 activated Wnt/β-catenin signaling and elevated the ALP expression and proliferation marker PCNA in DP cells. KY19382 also increased hair length in *ex vivo* cultured mouse vibrissa and human hair follicles and induced hair regrowth in mice. Moreover, KY19382 significantly promoted the generation of *de novo* hair follicles as shown by WIHN and hair patch assays.

Conclusion and Implications: These results indicate that KY19382 is a potential therapeutic drug that exhibits effective hair regeneration ability via activation of the Wnt/β-catenin signaling for alopecia treatments.

Keywords: Dermal papilla cells, Wnt/β-catenin signaling, GSK-3β, CXXC5, neogenesis

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Abbreviations: DP, dermal papilla; I3O, indirubin-3'-monoxime; CXXC5, CXXC-type zinc finger protein 5; Dvl, Dishevelled; WIHN, wound-induced hair follicle neogenesis; ALP, alkaline phosphatase; MNX, minoxidil; GSK-3β, glycogen synthase kinase-3β; PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; NBT/BCIP, nitro-blue tetrazolium, and 5-bromo-4-chloro-3'-indoly-phosphate; pNPP, p-nitrophenyl phosphate; IHC, immunohistochemistry; H&E, hematoxylin and eosin;

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What is already known

- Wnt/β-catenin signaling plays an important role in hair regrowth and regeneration.
- CXXC5 inhibits hair regeneration by suppressing the Wnt/β-catenin signaling pathway.

What this study adds

- Newly discovered small molecule promotes hair regeneration by simultaneously inhibiting GSK-3β and blocking CXXC5-Dvl interaction.
- This molecule induces HFSC activation and hair induction marker, unlike commercial hair growth-promoting agents.

What is the clinical significance

- Drugs enhancing WIHN are commercially unavailable but need to be developed for alopecia treatment.
- Dual targeting of GSK-3β and CXXC5-Dvl interaction could be a new treatment for baldness.

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1. INTRODUCTION

Recent reports show a significant increase in the number of people with alopecia (Jang, Son et al., 2013). In the skin of alopecia patients, many hair follicles overstay in their resting stage (Eckert, Church et al., 1968; Pratt, King et al., 2017) in which dermal papilla (DP) cells and keratinocytes, the two key players of hair cycle regulation, are inactive (Alonso & Fuchs, 2006; Botchkarev & Kishimoto, 2003; Sennett & Rendl, 2012). Various factors cause the inactivation of these cells and the inactivation leads to miniaturized hair follicles, resulting in hair loss (Kligman, 1959; Price, 1999). Even though researchers have focused on developing drugs to cure alopecia, only two drugs, minoxidil (MNX) and finasteride, have been approved by the US Food and Drug Administration for clinical treatment of androgenic alopecia (Libecco & Bergfeld, 2004; Linas & Nies, 1981; Price, 1999). Although both drugs effectively promote hair growth, neither can initiate hair follicle neogenesis effectively due to the complicated process of hair regeneration (Paus, 2006).

Wnt/β-catenin signaling pathway plays an important role in hair morphogenesis, growth initiation and regeneration (Andl, Reddy et al., 2002; Huelsken, Vogel et al., 2001; Ito, Yang et al., 2007; Kishimoto, Burgeson et al., 2000). The activated Wnt/β-catenin signaling pathway initiates embryonic hair formation in the epidermis and promotes the formation of dermal condensates (Andl, Reddy et al., 2002; Huelsken, Vogel et al., 2001). In addition, DP cells require activation of this pathway to initiate hair-inducing activity that prolongs the anagen phase (Kishimoto, Burgeson et al., 2000). The hair-inducing ability of DP cells can be confirmed by analyzing the expression of alkaline phosphatase (ALP) (Iida, Ihara et al., 2007; Lee, Yoon et al., 2012).Recent studies showed that the activation of Wnt/β-catenin signaling could recover ALP expression in long-term cultured primary DP cells (Yamauchi & Kurosaka, 2009).

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Hair growth-promoting drugs targeting Wnt/β-catenin signaling are not currently available. However, small molecules are needed that, unlike MNX, can activate this pathway to enhance hair follicle neogenesis (Lee, Seo et al., 2017).

KY19382 is one of the newly synthesized analogs of indirubin-3'-monoxime (I3O), a glycogen synthase kinase-3β (GSK-3β) inhibitor, and has a significant ability to activate the Wnt/β-catenin signaling (Choi, Kim et al., 2019). Indirubin is an active ingredient of the indigo plant, Danggui Longhui Wan, used as traditional anti-leukemia medicine (Xiao, Hao et al., 2002). I3O is known as a Wnt/β-catenin activator that stabilizes β-catenin via inhibition of GSK-3β, and has been shown to accelerate bone growth and inhibit adipogenesis (Choi, Cho et al., 2014; Zahoor, Cha, et al., 2014a; Zahoor, Cha, et al., 2014b). Furthermore, we recently found that KY19382 elongated tibial length by inactivating GSK-3β and blocking the binding of CXXC-type zinc finger protein 5 (CXXC5) and Dishevelled (Dvl) (Choi, Kim et al., 2019). Moreover, PTD-DBM, a peptide that interferes with CXXC5-Dvl interaction via binding to the PDZ domain of Dvl, stimulated wound-induced hair follicle neogenesis (WIHN) and hair regrowth (Lee, Seo et al., 2017). However, this peptide is limited for routine application due to its cost and stability.

In this study, we selected KY19382 as an optimal compound that can stimulate hair growth. KY19382 activated the Wnt/β-catenin signaling higher than I3O and showed low cytotoxicity in human DP cells. The KY19382 elongated rodent vibrissa and human hair shaft and mouse dorsal hair. Moreover, KY19382 significantly induced the hair follicle neogenesis as shown in hairless mice injected with dermal cells and keratinocytes and wounded mice treated with KY19382. Overall, KY19382 is an effective Wnt/β-catenin signaling activator that can be used for the treatment of alopecia with high efficacy and safety.

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2. METHODS

2.1 Cell culture and reagents

Primary human DP cells are described in our previous study (Shin, Kwack et al., 2010). Primary human DP cells from passages 2-7 were used in this study. The cells were cultured in Dulbecco's low glucose modified Eagle's medium (Hyclone, Pittsburgh, USA) supplemented with 10% fetal bovine serum (Gibco, Gaithersburg, USA), 1% antibiotic-antimycotic (Gibco, Gaithersburg, USA), 1 ng/ml bFGF (Peprotech, Princeton, USA), and 5 μg/ml insulin (Gibco, Gaithersburg, USA) at 37°C in a humidified atmosphere of 5% CO_2.

A rat vibrissa immortalized DP cell line was donated by the Skin Research Institute of the Amore Pacific Corporation R&D Center (Yongin, Korea). HEK293 Wnt/β-catenin signaling reporter cell (HEK293 cells (ATCC Cat# CRL-1573, RRID: CVCL_0045) containing a chromosomally integrated TOPflash reporter) is described in our previous study (Choi, Kim et al., 2019). Rat DP or HEK293 reporter cells, were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Gaithersburg, USA) containing 10% (v/v) fetal bovine serum (Gibco, Gaithersburg, USA) and 100 U/ml penicillin/streptomycin (Gibco, Gaithersburg, USA) at 37°C in a humidified atmosphere of 5% CO_2. All compounds were dissolved in dimethyl sulfoxide (DMSO) (Sigma Aldrich, St. Louis, USA), and the cells were incubated with 0.1% (v/v) DMSO or each compound in DMEM.

2.2 Cell viability assay

HEK293 reporter, human DP, or rat DP cells were seeded in 24-well plates, and treated for 24 hours for HEK293 reporter cells or for 48 hours in DP cells with 0.1% (v/v) DMSO or 100 μM MNX (Tokyo Chemical Industry Co., Tokyo, Japan). The identically grown cells were also

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treated with KY19382 or I3O as the concentrations shown in figure 1a, supplementary figure 1a or supplementary figure 2a. Cell viability was measured by the Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega, Madison, USA) as described in the manufacturer's instruction. The luminescence activities were measured using the FLUOstar OPTIMA luminometer (BMG Labtech, Offenburg, Germany).

2.3 Luciferase reporter assay

HEK293 reporter cells were seeded in 24-well plates, and the cells were treated 24 hours with the vehicle (0.1% (v/v) DMSO) or indicated concentrations of KY19382 or I3O in figure 1a. The cells were washed with cold phosphate-buffered saline (PBS) and lysed in 55 µl of 1x lysis buffer (Promega, Madison, USA). The cell lysates were centrifuged at 15,920x g at 4°C for 15 minutes. Thirty microliters of each supernatant were transferred to 96-well plates, and 15 µl luciferin was added. The luciferase activity was measured at 485 nm using a FLUOstar OPTIMA luminometer (BMG Labtech, Offenburg, Germany).

2.4 Immunoblotting

The cells or tissue were washed twice with cold PBS and lysed in RIPA buffer (150 mM NaCl; 10 mM Tris, pH 7.2; 0.1% SDS; 1% Triton X-100; 1% sodium deoxycholate; 5 mM EDTA). The lysates were centrifuged at 15,920x g at 4°C for 30 minutes. The equal amounts of protein (20 µg) were separated on 8-10% SDS PAGE gels and transferred onto PROTRAN nitrocellulose membranes (Schleicher and Schuell Co., Keene, USA). Precision Plus Protein Standards (161-0373, Bio-rad, Hercules, USA) was used as a molecular weight marker. After blocking with 5% skim milk for 1 hour at room temperature, each membrane was blotted with the following primary antibodies: mouse anti-β-catenin (BD Biosciences Cat# 610154, RRID:

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AB_397555; 1:3000, Lexington, USA), mouse anti-α-tubulin (Cell Signaling Technology Cat# 3873, RRID: AB_1904178, 1:4000, Danvers, USA), rabbit anti-Erk1/2 (Cell Signaling Technology Cat# 9102, RRID: AB_330744, 1:1000, Danvers, USA), rabbit anti-p-GSK-3β (S9) (Cell Signaling Technology Cat# 9336, RRID: AB_331405, 1:1000, Danvers, USA), mouse anti-PCNA (Santa Cruz Biotechnology Cat# sc-56, RRID: AB_628110; 1:500, Dallas, USA), rabbit anti-fgf9 (Abcam Cat# ab71395, RRID: AB_2103075, 1:1000, Cambridge, USA) and rabbit anti-cytokeratin 17 (Abcam Cat# ab53707, RRID: AB_869865, 1:1000, Cambridge, USA) at 4ºC overnight. Each membrane was blotted with horse-radish peroxidase-conjugated anti-mouse (Cell Signaling Technology Cat# 7076, RRID: AB_330924, 1:3000, Danvers, USA) or anti-rabbit (Bio-Rad Cat# 1706515, RRID: AB_11125142, 1:3000, Hercules, USA) IgG secondary antibody. The dilutions of antibody were maintained at -20°C and re-used up to five times. The blots were visualized using enhanced chemiluminescence (Amersham Bioscience, Buckinghamshire, UK) and a luminescent image analyzer (LAS-4000; Fujifilm, Tokyo, Japan). Immunoblot analyses were performed in accordance with the BJP guidelines (Alexander, Roberts et al., 2018).

2.5 Immunocytochemistry

Human or rat DP cells were seeded in a 12-well plate on coverslips. The cells were incubated with 0.1% (v/v) DMSO as the vehicle, 1 or 5 μM KY19382, or 100 μM MNX for 48 h. Cultured cells were washed twice with cold PBS and were fixed in 4% paraformaldehyde (Wako, Osaka, Japan) or 10% formalin (Sigma Aldrich, St. Louis, USA) for 15 min at room temperature, and then were washed with PBS and permeabilized with 0.2% Triton X-100 for 15 min.

After blocking with 5% BSA in PBS for 30 min at room temperature, the cells were blotted with primary antibody: rabbit anti-β-catenin (Abcam Cat# 16051, RRID: AB_443301, 1:50,

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Cambridge, USA) overnight at 4°C. After washing with PBS, the cells were blotted with Alexa Fluor 488-conjugated goat anti-mouse antibody (Thermo Fisher Scientific Cat# A-11001, RRID: AB_2534069, 1:300, Waltham, USA) or Alexa Fluor 555-conjugated goat anti-rabbit antibody (Thermo Fisher Scientific Cat# A-21428, RRID: AB_2535849, 1:300, Waltham, USA) for 1 h at room temperature and counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma Aldrich, St. Louis, USA) for 10 min at room temperature. Images were taken using an LSM510 confocal microscope (Carl Zeiss Inc., Germany). The fluorescence intensity was quantified using Zen software V3.1 software (ZEN Digital Imaging for Light Microscopy, RRID: SCR_013672, Germany).

2.6 Alkaline phosphatase staining

For ALP staining in cells, human or rat DP cells were seeded in a 12-well plate on coverslips. Cells were incubated with 0.1% (v/v) DMSO as the vehicle, 1 or 5 µM KY19382, or 100 µM MNX for 48 hours and washed twice with cold PBS and were fixed in formalin for 15 minutes at room temperature. Then cells were incubated with nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate solution (NBT/BCIP solution, Sigma Aldrich, St. Louis, USA). The reaction was stopped by washing with PBS. Dark blue staining was a positive signal for ALP.

For ALP staining of tissues, 20-µm cryosections were dried for 4 hours, then fixed in 4% paraformaldehyde for 5 minutes. After being washed with PBS, the sections were incubated in NBT/BCIP solution (NBT/BCIP tablets, Roche Diagnostics, Rotkreuz, Switzerland) for 30 minutes. The slides were counterstained with the nuclear fast red solution (Sigma Aldrich, St. Louis, USA) for 30 seconds, washed in distilled water, and dried for 2 hours. The slides were incubated in 100% xylene for 30 seconds, then mounted using Permount (Thermo Fisher Scientific, Waltham, USA).

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For whole-mount ALP staining, the wounded skin tissues were incubated 5 mM EDTA in PBS at 37 ºC for 6 hours. The dermis was separated under the stereomicroscope (SMZ 745T; Nikon, Tokyo, Japan). The dermis was fixed in 4% paraformaldehyde for 10 minutes, washed with PBS, and then incubated in NBT/BCIP solution (NBT/BCIP tablets, Roche Diagnostics, Rotkreuz, Switzerland) for 10 minutes. The whole mount images of dermis were taken by using a stereomicroscope (Nikon, Tokyo, Japan). The number of ALP-positive neogenic follicles was measured by counting dark blue dots.

2.7 ALP activity assay

Human or rat DP cells were seeded in 24-well plates and incubated with 0.1% (v/v) DMSO as the vehicle control, 1 or 5 μM KY19382, or 100 μM MNX for 48 hours. The cells were washed twice with cold PBS and lysed with 55 μl 1x reporter lysis buffer (Promega, Madison, USA) per well. Cell lysates were centrifuged at 10,000x g at 4°C for 30 minutes. Thirty microliters of each supernatant were incubated with 30 μl of p-nitrophenyl phosphate (pNPP) liquid substrate (Sigma Aldrich, St. Louis, USA) for 1 hour. ALP activity was measured at 405 nm using the FLUOstar OPTIMA luminometer and normalized by the protein concentration from the Bradford assay (Bio-rad, Hercules, USA).

2.8 siRNA preparation and transfection

The human DP cells were transfected with siRNA or the negative control (Bioneer, Daejeon, Korea) using Lipofectamine Plus (Invitrogen, Carlsbad, USA) in serum-free Opti-MEM (Gibco, Gaithersburg, USA) according to the manufacturer's instructions at a final concentration of 100 nM. The siRNA sequences targeting β-catenin were 5'-

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GAAACGGCTTTCAGTTGAG-3' and 5'-AAACTACTGTGGACCACAAGC-3' (Bioneer, Daejeon, Korea). Twelve hours after transfection with β-catenin siRNA, the human DP cells were treated with 0.1% DMSO as the vehicle or 5 μM KY19382 for 48 hours. ALP assay and immunoblotting were performed to examine ALP activity changes and confirm the transfection efficiency of β-catenin siRNA.

2.9 *Ex vivo* vibrissa follicle culture

Mouse vibrissa follicles were isolated from a C57BL/6N mouse. After euthanizing the mouse with CO_2 gas, anagen follicles were isolated for organ culture under a stereomicroscope (Nikon, Tokyo, Japan). The isolated follicles were placed in 500 μl DMEM supplemented with 100 U/ml penicillin/streptomycin (Gibco, Gaithersburg, USA) and 12.5 μg/ml gentamicin (Gibco, Gaithersburg, USA) in 24-well plates. The vibrissa follicles were treated with 0.1% (v/v) DMSO as the vehicle control, 5 μM KY19382, or 100 μM MNX. The culture medium was changed every 2 days.

Rat vibrissa follicles were isolated from a 21-day-old male Wistar rat (Philpott, Green et al., 1992). After euthanizing the rat with CO_2 gas, anagen follicles were separated under a stereomicroscope (Nikon, Tokyo, Japan). The rat vibrissa follicles were treated with 1 or 5 μM KY19382 or 0.1% DMSO as the vehicle control. The culture medium was changed every 4 days. Rat experiments have been approved by the Institutional Animal Care and Use Committee (IACUC) of Yonsei University (IACUC-A-201407-260-01).

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2.10 Immunohistochemistry (IHC)

Skin tissues were fixed in 4% paraformaldehyde or 10% formalin overnight at 4°C. Paraffin sections were deparaffinized and rehydrated. The slides were autoclaved or microwaved for 15 minutes in 10 mM sodium citrate buffer for antigen retrieval. The samples were pre-incubated in PBS and then blocked with 5% BSA in PBS for 30 minutes at room temperature. The samples were incubated overnight at 4°C with the following primary antibodies: rabbit anti-β-catenin (Abcam Cat# 16051, RRID: AB_443301, 1:100, Cambridge, USA), mouse anti-keratin 15 (Abcam Cat# ab80522, RRID: AB_1603675, 1:200, Cambridge, USA), anti-PCNA (Santa Cruz Biotechnology Cat# sc-56, RRID: AB_628110; 1:500, Dallas, USA), anti-Ki67 (Abcam Cat# ab15580, RRID: AB_443209, 1:500, Cambridge, USA), anti-fgf9 (Abcam Cat# ab71395, RRID: AB_2103075, 1:200, Cambridge, USA) and anti-cytokeratin 17 (Abcam Cat# ab53707, RRID: AB_869865, 1:400, Cambridge, USA). After washing with PBS, the slides were incubated with anti-mouse Alexa Fluor 488 (Thermo Fisher Scientific Cat# A-11001, RRID: AB_2534069, 1:500, Waltham, USA) or anti-rabbit Alexa Fluor 555 (Thermo Fisher Scientific Cat# A-21428, RRID: AB_2535849, 1:500, Waltham, USA) conjugated secondary antibody for 1 hour at room temperature and counterstained with DAPI (Sigma Aldrich, St. Louis, USA) for 10 minutes. The images were taken using an LSM510 confocal microscope (Carl Zeiss Inc., Germany). The fluorescence intensity was measured using Zen software V3.1 software (ZEN Digital Imaging for Light Microscopy, RRID: SCR_013672, Germany). IHC analyses were conducted by following the BJP guidelines (Alexander, Roberts et al., 2018).

2.11 Human hair follicle culture

The detailed method is described in the previous paper (Kang, Bak et al., 2019). Hair follicles were isolated from the occipital nonbalding scalps of androgenic alopecia male patients from

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2 persons during hair transplantation. Informed written consent was obtained from all patients. The Medical Ethical Committee of the Kyungpook National University Hospital approved all the procedures. The study was performed following the Declaration of Helsinki principles. The hair follicles were incubated in Williams' medium E without phenol red (Sigma Aldrich, St. Louis, USA) at 37 °C in a humidified atmosphere of 5% CO_2. The human follicles were incubated with 0.1% DMSO as vehicle control, 5 μM KY19382, or 100 μM MNX.

2.12 X-gal staining

Axin2$^{LacZ/+}$ Wnt/β-catenin signaling reporter mouse was purchased from Jackson Laboratory (Bar Harbor, USA). Vibrissa follicles were fixed in 0.4% paraformaldehyde for 3 hours and 0.2% glutaraldehyde for 15 minutes, then washed with PBS. The tissues were incubated in X-gal solution (Thermo Fisher Scientific, Waltham, USA) at 37 ºC overnight. Sky blue staining represents a positive signal for X-gal.

2.13 Compliance with requirements for studies using animal

The protocol and group size for animal studies referred to previous studies (Lee, Seo et al., 2017; Lee, Yoon et al., 2012). All protocols for anesthesia methods, ex vivo vibrissa experiments, hair re-growth experiments, WIHN experiments, and hair reconstitution assays were approved by the Institutional Animal Care and Use Committee (IACUC) of Yonsei University (IACUC-A-201407-260-01, IACUC-A-201712-672-03, IACUC-A-201804-721-02, IACUC-A-201807-761-02, IACUC-A-201811-823-03, IACUC-A-201901-857-01, IACUC-A-201909-954-01, IACUC-A-201907-929-01). Animal research is stated in

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compliance with the ARRIVE guidelines (Percie du Sert, Hurst et al., 2020) and recommendations made by the British Journal of Pharmacology 2020. Mice were bred in a micro-ventilation cage system with a computerized environmental control system (Threeshine Inc, Seoul, Korea). Mice had free access to food and water. Temperature of the room was maintained at 24°C with a relative humidity of 40-70% and 12 h light/dark cycle. The mice were housed with a standard diet and bedding from the Central Lab. Animal Inc. (Seoul, South Korea). Mice were housed with no more than 5 animals per cage. The data and statistics of animal experiments were analyzed in compliance with the British Journal of Pharmacology recommendations on experimental design and analyses in pharmacology (Curtis, Alexander et al., 2018). Group sizes of animal experiments included more than 5 animals in at least three independent experiments.

Considering the 3Rs (replacement, refinement, or reduction) of animal use, we first conducted preliminary experiments to confirm the effect of KY19382 on hair growth through DP cell culture or *ex vivo* follicle culture, and then conducted minimal animal experiments under optimal conditions.

2.14 *In vivo* hair growth test

Specific pathogen-free six-week-old C57BL/6N or C3H wild-type male mice (approximately 22g) were purchased from Koatech Co. (Gyeonggido, Korea) or Orient Bio Co. (Gyeonggido, Korea) and acclimated for 1 week. After anesthesia with 2,2,2-tribromoethanol (Sigma Aldrich, St. Louis, USA) through 400 mg/kg IP injection, the hairs on the backs of 7-week-old mice, whose hair follicles were in the telogen phase, were shaved with a hair clipper. The mice were randomly separated into 4 groups following the BJP guidelines. For each of the mice, 300 μl of each drug was applied topically daily at an appropriate concentration (as described in figure

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legends) for up to 14 or 28 days. The indicated concentrations of KY19382 were dissolved in polyethylene glycol 400 (PEG400) (Sigma Aldrich, St. Louis, USA; vehicle 1), and 100mM minoxidil was dissolved in 50% (v/v) ethanol (Duksan Pure Chemicals Co., Gyeonggido, Korea), 30% water, and 20% propylene glycol (Junsei Chemical Industry Co., Tokyo, Japan) (vehicle 2). After the experiments were completed, the mice were euthanized using CO_2 gas.

2.15 Hematoxylin and eosin staining (H&E)

The tissues were dehydrated, paraffinized, embedded in paraffin, and sliced into 4 μm thickness. The paraffin sections were rehydrated through xylene and graded ethanol series. The slides were incubated in hematoxylin for 5 minutes and eosin for 1 minute. The number of follicles was calculated by counting the hair follicles in H&E staining images. Dermal thickness was measured by using Image J (ImageJ, RRID: SCR_003070) software V1.48.

2.16 Wound-induced hair follicle neogenesis assay

Specific pathogen-free three-week-old C57BL/6N wild-type male mice (approximately 11g) were allowed to adapt to their new environment for 3 days. After anesthesia with 2,2,2-tribromoethanol (400 mg/kg, IP), 1 cm^2 full-thickness wounds were generated on the backs of the mice under aseptic conditions. The mice were randomly distributed into 4 groups following the BJP guidelines. 20 μl of each drug was applied to the wounds daily for up to 14, 25, or 40 days. After the studies were completed, the mice were euthanized using CO_2 gas.

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2.17 Hair patch assay

Epidermal and dermal cells were isolated from neonatal C57BL/6N mice. The skin of neonatal mice was peeled and digested with 0.25% β-trypsin to separate the epidermis and dermis. The separated dermal cells were seeded in a 100 mm culture dish and incubated with 5 μM KY19382 or 0.1 % DMSO as vehicle control for 72 hours. 1×10^6 epidermal and 1.5×10^6 dermal cells were injected subcutaneously into hairless mice. After the experiments were completed, the mice were euthanized using CO_2 gas.

2.18 Data and analyses

In vivo and *in vitro* experiments were designed to establish equal size, blinding, and randomization. The statistical analyses were performed only for experiments where group sizes (n) ≥ 5. All group sizes represent the numbers of experimental independent values, and these independent values were used to evaluate statistical analyses.

All statistical data were presented as means ± SEM. Comparisons between two unpaired treatment groups were tested by Student's t-test. One-way ANOVA with Tukey's test was performed for comparisons between multi-group studies, post hoc tests were conducted only if the F in ANOVA achieved the necessary statistical significance level ($P < .05$). There was no significant variance inhomogeneity. Prism software V5.01 (GraphPad Prism, RRID: SCR_002798, CA, USA) was used for statistical analyses.

The threshold for statistical significance was set at $P < .05$. $P < .05$ was regarded as statistically significant. Statistical significance is indicated in the figures as follows: NS, not significant; * $P < 0.05$.

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To reduce unwanted sources of variation, data normalization was performed. For the experimental results including cell viability, quantification of images, ALP activity, TOPflash activity, elongation of vibrissa or hair follicles, and hair weight, each value was divided by the mean of the control values and indicated in the form of "% or relative." For these results, "fold mean of the controls." was labeled in the Y-axis of the figures. No data values were excluded in the any test during statistical analyses. The data and statistical analyses were performed in compliance with the recommendations and requirements of British Journal of Pharmacology on experimental design and analyses in pharmacology (Curtis et al., 2018).

2.19 nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding, Sharman et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander, Kelly et al., 2019).

3. RESULTS

3.1 KY19382 induces ALP activity by activating Wnt/β-catenin signaling in human DP cells

KY19382, a newly synthesized small molecule derived from I3O, was screened by its capability to interfere with the CXXC5-Dvl protein-protein interaction, followed by activation of Wnt/β-catenin signaling (Choi, Kim et al., 2019). KY19382 showed significantly higher Wnt activity than I3O in a concentration-dependent manner in HEK293 reporter cells with low cytotoxicity (Supplementary figure 1a and 1b). To determine the highest concentration of KY19382 without toxic effects in DP cells, cell viability was preliminary measured in multiple concentrations in rat DP cells. Cell viability was not significantly affected at 1 and 5 μM

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KY19382 but decreased at 10 μM KY19382 for rat DP cells (Supplementary figure 2a). In addition, 1 and 5 μM KY19382 did not show any toxicity in human DP cells (Figure 1a). Thus, 1 and 5 μM KY19382 was used for subsequent *in vitro* studies.

To determine whether KY19382 activates Wnt/β-catenin signaling via GSK-3β inhibition, we examined the levels of β-catenin and p-GSK-3β (S9), an inactive form of GSK-3β, in DP cells. When treated with KY19382, the expression levels of β-catenin, p-GSK-3β (S9), and proliferation marker, PCNA were increased in DP cells (Figure 1b and supplementary figure 2b). The upregulation of Wnt/β-catenin signaling was further confirmed by cytochemical analyses of DP cells treated with KY19382 (Figure 1c and supplementary figure 2c). Quantitative measurements of both total and nuclear β-catenin intensities shown that the Wnt/β-catenin signaling was activated by KY19382 treatment in DP cells (Figure 1d, 1e and supplementary figure 2d, 2e). To assess the effect of KY19382 on ALP activity induction, we performed ALP staining and ALP activity assays in DP cells. KY19382 treatment resulted in higher ALP staining intensity and activity compared to those in the vehicle-treated group (Figure 1f, 1g and supplementary figure 2f, 2g).

The effect of Wnt/β-catenin signaling on KY19382-induced ALP activity was confirmed using human β-catenin siRNA transfection on human DP cells. The knock-down effect of β-catenin siRNA transfection was verified using immunoblotting analyses (Supplementary figure 2h). KY19382-induced ALP expression and activity were abolished by transfection with β-catenin siRNA in human DP cells; however, this inhibitory effect was not shown in cells transfected with a control siRNA (Figure 1h and 1i).

Overall, these data showed that KY19382 significantly activated Wnt/β-catenin signaling in both human and rat DP cells with low cytotoxicity. In addition, KY19382 treatment elevated ALP activity by activating Wnt/β-catenin signaling.

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3.2 KY19382 promotes hair growth and activates the Wnt/β-catenin signaling in *ex vivo*-cultured human scalp hair and rodent vibrissa follicles

To determine whether KY19382 promotes hair follicle growth, we treated human scalp hair and rodent vibrissa follicles with 0.1% DMSO as vehicle control, KY19382 or MNX in *ex vivo*. KY19382 treatment on human hair follicles for 3 days, mouse vibrissa follicles for 6 days and rat vibrissa follicles for 12 days significantly promoted follicle elongation. (Figure 2a and 2e and supplementary figure 3b).

To confirm the effect of KY19382 on the activation of Wnt/β-catenin signaling in an *ex vivo*-cultured system, we performed IHC analyses. An increase in β-catenin was observed in human follicles and rat vibrissa follicles (Supplementary figure 3a, 3c and 3d). In addition, both the β-catenin and proliferation marker, Ki67, increased in the KY19382-treated mouse vibrissa follicles compared to those in vehicle-treated follicles (Figure 2b). Moreover, increased ALP expression was detected in cultured mouse vibrissa follicles treated with KY19382, demonstrating the hair-inducing effect of KY19382 (Figure 2c). Correspondingly, LacZ expression was highly increased at the base of mouse vibrissa follicles from $Axin2^{LacZ/+}$ mice after KY19382 treatment, showing its strong effect on Wnt/β-catenin signaling activation (Figure 2d).

In summary, β-catenin, Ki67, ALP, and Axin2-LacZ expression levels were concomitantly increased at the hair bulb when treated with KY19382, suggesting that KY19382 accelerated follicle growth and activated Wnt/β-catenin signaling *ex vivo* for both rodent and human.

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3.3 KY19382 promotes hair regrowth in mice

To find the optimum concentration of KY19382 *in vivo*, a preliminary experiment was conducted in which various concentrations of KY19382 were topically treated daily on the shaved dorsal skins of 7-week-old C3H mice for 28 days. The most significant hair regrowth effect was observed by treatment of 0.5 mM KY19382 (Supplementary figure 4a and 4b). To compare the effects of KY19382 and MNX on mouse hair regrowth, vehicle 1, vehicle 2, KY19382 or MNX was topically applied to the dorsal skins of 7-week-old C57BL/6N mice daily for 14 or 28 days. MNX was used as the positive control (Davies, Thornton et al., 2005; Lee, Yoon et al., 2012; Park, Park et al., 2015; Sun, Cui et al., 2013; Takahashi, Kamimura et al., 2003). After 28 days, KY19382 promoted hair regrowth more efficiently than MNX (Figure 3a). The more effective hair growth promotion effect by KY19382 than by MNX was also shown by measurement of the weight of newly growth hair (Figure 3b). Hematoxylin and eosin (H&E) staining showed that hair follicles in the control group were still in the telogen phase, but hair follicles of skin treated with KY19382 or MNX for 28 days entered the anagen phase (Figure 3c). The increment of dermal thickness and hair follicle numbers indicates the telogen-anagen transition (Lee, Seo et al., 2017). The more effective transition to anagen by KY19382 than MNX was shown by measurement of the relative hair follicle numbers and dermal thickness in H&E staining images (Figure 3d and 3e). To confirm KY19382-induced changes in proliferation or Wnt/β-catenin signaling in the bulge, IHC analyses were performed on skin tissues treated for 14 days. Proliferation markers, ki67 and PCNA, were specifically increased in keratin 15-positive bulge stem cells of the KY19382-treated group (Figure 3f and supplementary figure 4c). β-Catenin was also increased only in the KY19382-treated group in bulge stem cells (Figure 3f). Quantitative analyses suggested that the Wnt/β-catenin signaling in bulge stem cells was activated by KY19382 treatment (Figure 3g and 3h). Moreover, ALP, a critical marker for hair induction, was highly expressed in the DP cells of skins treated with

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KY19382 for 14 days (Figure 3i). Similarly, western blot analyses showed that the levels of β-catenin and PCNA were significantly increased in the KY19382-treated group (Figure 3j). These data collectively suggested that KY19382 promoted hair regrowth and increased markers for hair growth promotion, such as β-catenin, PCNA, and ALP more efficiently than MNX.

3.4 KY19382 enhances wound-induced hair follicle neogenesis

Considering that KY19382 stimulated hair growth *in vitro*, *ex vivo,* and *in vivo* systems via activation of Wnt/β-catenin signaling, the effect of KY19382 on WIHN was tested in mice.

To confirm this, we cut 1 cm^2 full-thickness wounds in 3-4-week-old C57BL/6N mice and applied the drugs daily for 14, 25, and 40 days after wounding. The KY19382-treated group increased the number of newly formed follicles compared to those in the vehicle- or MNX-treated group, as confirmed by whole-mount ALP staining (Figure 4a). The ALP-positive signals, indicated by dark blue dots, showed that the neogenic hair growth was induced by treatment of KY19382 (Figure 4b). In addition, H&E staining showed that KY19382 induced the formation of neogenic follicles 14 days post-wounding (Figure 4c). IHC analyses showed that keratin 17, a marker for intermediate filament keratin protein (Ito, Yang et al., 2007), was specifically increased in neogenic follicles of the KY19382-treated group (Figure 4d). β-Catenin and proliferation markers, ki67 and PCNA, were increased in the KY19382-treated group, especially in the neogenic follicle regions (Figure 4d and supplementary figure 4d). Quantitative measurements showed that the Wnt/β-catenin signaling activation and proliferation in neogenic follicles were elevated by KY19382 treatment (Figure 4e, 4f and 4h). Fgf9 involved in WIHN in wound fibroblasts (Lee, Seo et al., 2017) was also increased in the dermis of wounds treated with KY19382 (Figure 4d and 4g). The newly formed white hairs at the wound sites were found only in the KY19382-treated group (Figure 4i). Furthermore,

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western blot analyses confirmed that the markers associated with hair follicle neogenesis were increased in wounded skin treated with KY19382, but not MNX (Figure 4j). Taken together, KY19382 markedly induced WIHN and WIHN-related markers by significantly activating the Wnt/β-catenin pathway.

3.5 KY19382 promotes hair follicle neogenesis in patch assays

To investigate the therapeutic effect of KY19382, we utilized a hair patch assay system. Mouse dermal cells were cultured with 0.1% DMSO as vehicle control or 5 μM KY19382 for 72 hours before transplantation, then mixed with epithelial cells for injection into hairless mice.

After 14 days post-injection, reconstituted hair follicles were observed on the skin of hairless mice where cells were injected (Figure 5a). The magnified images showed that the number and density of neogenic hair follicles generated in the tissue injected with the KY19382-treated cells were significantly higher than those injected with the vehicle-treated control cells (Figure 5b). As quantitatively analyzed, the KY19382 treatment increased the number of newly formed hair follicles (Figure 5c). Moreover, the expression levels of β-catenin and Ki67 were greatly increased in neo-generated hair follicles induced by KY19382, as demonstrated by IHC analyses (Figure 5d). The intensity of nuclear β-catenin and the number of Ki67-positive cells in neogenic hair follicles were elevated by KY19382 (Figure 5e and 5f).

4. DISCUSSION

Currently, available drugs for treating alopecia are limited due to their inability to regenerate hair follicles. MNX and finasteride can promote hair growth when the hair follicle is present, but they are not effective in patients with severe alopecia (Libecco & Bergfeld, 2004; Messenger & Rundegren, 2004; Price, 1999; Rossi, Anzalone et al., 2016). Existing drugs that

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control the proliferation of hair cells can be difficult for treating patients with miniaturized or absent hair follicles (Han, Kwon et al., 2004). Therefore, we aimed to develop a drug that is effective in promoting hair regrowth and hair follicle neogenesis by inducing markers for hair induction such as ALP and activating hair follicle stem cells via the Wnt/β-catenin pathway.

Hair follicle neogenesis could be an important strategy for alopecia treatment (Ito, Yang et al., 2007). Previous studies have shown that hair follicle neogenesis highly depends on the activation of Wnt/β-catenin signaling in DP cells and keratinocytes, along with activation of hair follicle stem cells (Enshell-Seijffers, Lindon et al., 2010; Huelsken, Vogel et al., 2001; Ito, Yang et al., 2007; Waters, Richardson et al., 2007). Wnt/β-catenin signaling plays essential roles in maintaining the hair-inducing ability of DP cells and promoting hair follicles to the anagen phase (Andl, Reddy et al., 2002; Kishimoto, Burgeson et al., 2000; Sick, Reinker et al., 2006). In addition, previous literature has suggested that Wnt/β-catenin signaling activators, such as valproic acid (Lee, Yoon et al., 2012), *Aconiti ciliare tuber* extract (Park, Moon et al., 2012) and *Malva verticillata* seed extract (Lee, Choi et al., 2016), are potential candidates for alternative hair growth treatments as they induce the expression of hair inducing markers in DP cells. Therefore, it is important to use Wnt/β-catenin signaling activators that activate both hair induction markers and stem cells to promote hair growth and regeneration effectively.

Although direct Wnt/β-catenin signaling activators such as valproic acid promote hair growth, but they fail to sustain hair growth and often show marginal effects in clinical tests (Jo, Choi et al., 2013; Jo, Shin et al., 2014). This marginal and limited effect may be attributed to functions of negative feedback regulators such as CXXC5 or DKK1 (Kwack, Kim et al., 2012; Lee, Seo et al., 2017). We found that CXXC5, a negative feedback regulator of Wnt/β-catenin signaling, is specifically increased in the miniaturized follicles of bald scalps, and *CXXC5* knock out mice exhibited enhanced hair growth (Lee, Seo et al., 2017). PTD-DBM, a peptide that interfered with CXXC5-Dvl protein interaction, enhanced hair growth, and the

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combinatory treatment of PTD-DBM and valproic acid synergistically increased hair growth and the WIHN (Lee, Seo et al., 2017). Similarly, KY19382, which strongly activated the Wnt/β-catenin signaling via interference of the CXXC5-Dvl interaction and inhibited GSK-3β (Choi, Kim et al., 2019), critically enhanced hair re-growth as well as WIHN *in vivo*. The increased CXXC5 in bald scalps and the effectiveness of PTD-DBM or KY19382 on hair growth in mice suggest the potential use of KY19382 as a clinical treatment for hair loss.

In normal physiology, the Wnt/β-catenin pathway plays roles in tissue regeneration including neogenic hair growth, but its aberrant activation by mutations such as loss of function mutation of *APC* and gain of function mutation of *β-catenin* (non-degradable form as a protein). often results in cancer (Fodde, 2002; Li, Kim et al., 2004). Our approach for activation of the Wnt/β-catenin pathway using KY19382 is not via direct activation, but via the release of the negative feedback mechanism through interference of the Dishevelled binding function of CXXC5. Therefore, we suggested that this approach activating the Wnt/β-catenin pathway by blocking the negative feedback mechanism would be safe, and this is supported by successful FDA approval of the antibody against Sclerostin, a LRP5/6 receptor binding protein inhibiting the Wnt/β-catenin pathway (Shakeri & Adanty, 2020).

In this study, we confirmed that the levels of β-catenin, p-GSK3β (S9), and proliferation marker, PCNA, were increased in human DP cells treated with KY19382. Increased ALP activity after KY19382 treatment suggested that KY19382 increased the hair inducing ability in human DP cells. Compared with the vehicle-treated group, mouse vibrissa follicles treated with KY19382 for 6 days significantly promoted elongation of the hair shaft. Furthermore, KY19382 increased the length and expression levels of β-catenin in human hair follicles. The levels of β-catenin, Ki67, and PCNA were increased-in the keratin 15-positive bulge of mouse skin treated with KY19382, suggesting a positive effect of KY19382 on the activity of hair follicle stem cells. Moreover, KY19382 regenerated a number of neogenic follicles in the

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WIHN assay, and histological images showed higher expression levels of β-catenin, proliferation markers, and markers for hair follicle neogenesis, fgf 9 and keratin 17. These results indicate that KY19382 treatment may be a possible therapy for baldness. In the hair patch assay, KY19382 regenerated a greater number of neogenic hair follicles, and histological evaluation revealed higher expression levels of β-catenin and Ki67 than control, demonstrating that pretreatment with KY19382 enhanced the hair-inducing ability of dermal cells. Therefore, our results suggest that KY19382 may be useful for the treatment of hair loss and baldness via its effective dual-targeting ability to inhibit both GSK-3β and CXXC5-Dvl interactions.

ACKNOWLEDGEMENTS

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean Government (MSIP) (2019R1A2C3002751, 2020M3E5E2040018). Y.C RYU was supported by a Brain Korea 21 (BK21) Plus studentship from the NRF.

CONFLICT OF INTEREST

K.Y.C is the CEO of CK Biotech Inc. (Seoul, Korea), which has a license to develop and use the compounds disclosed in the publication. The authors have no further conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

Y.C.R., D.L., J.S., and J.P. performed and analyzed the experiments. Y.K., S.C., and S.S.B. helped in vitro and in vivo experiments. Y.C.R., J.S., S.L., and K.C. wrote the manuscript. Y.K.S., S.L., and K.C. supervised the study.

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DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This declaration acknowledges that this paper adheres to the principles for transparent and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analyses, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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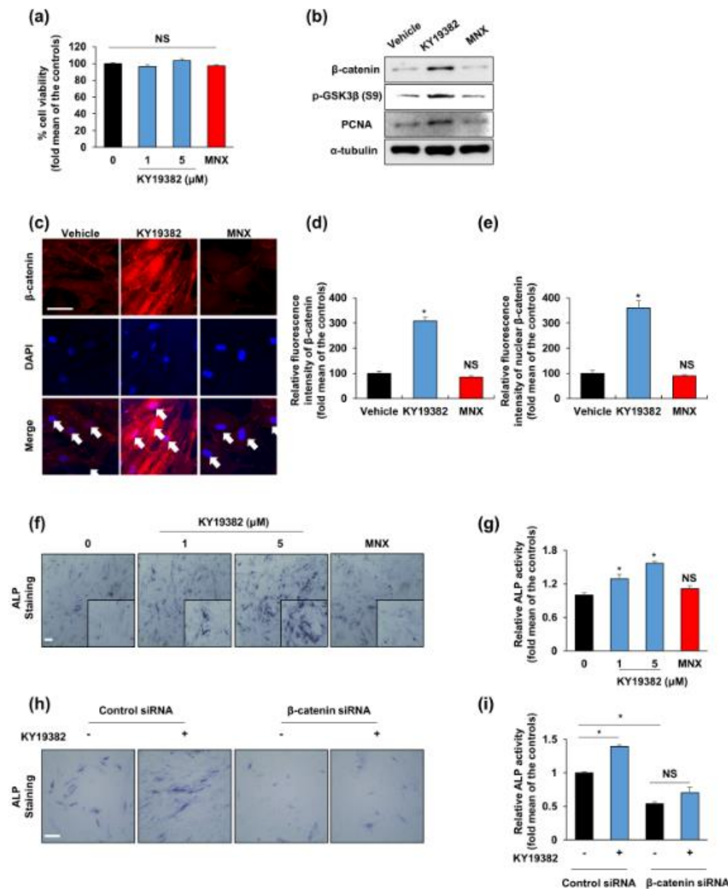

Figure 1. KY19382 increases hair induction activity via activation of the Wnt/β-catenin pathway. (a) Cell viability of human DP cells treated with vehicle (0.1 % (v/v) DMSO), 100 μM MNX, or the marked concentrations of KY19382 for 48 hours (n=6). (b-c) Human DP cells were treated with vehicle (0.1% DMSO), 5 μM KY19382, or 100 μM MNX for 48 hours. (b) Immunoblotting analyses for β-catenin, α-tubulin, p-GSK3β, and PCNA was analyzed using human DP cells. (c) Immunocytochemical staining for β-catenin. Nuclei were counterstained with DAPI. Arrows indicate nuclei. (d-e) Quantitative measurements of the total and nuclear β-catenin intensities of human DP cells (n=12 taken in 3 different images). (f-g) Human DP cells were treated with vehicle (0.1% DMSO), 100 μM MNX, or the marked concentrations of KY19382 for 48 hours. (f) ALP staining and (g) ALP activity test (n=5). (h-i) Human DP cells were transfected with β-catenin siRNA or negative control for 12 hours. After transfection, vehicle (0.1% DMSO) or 5 μM KY19382 was treated to human DP cells for 48 hours. (h) Transfected cells were subjected to ALP staining. (i) ALP activity was quantified (n=6). Scale bars = 100 μm. Values are expressed as means ± SEM (* $P < 0.05$, significant differences as indicated; NS, not significant).

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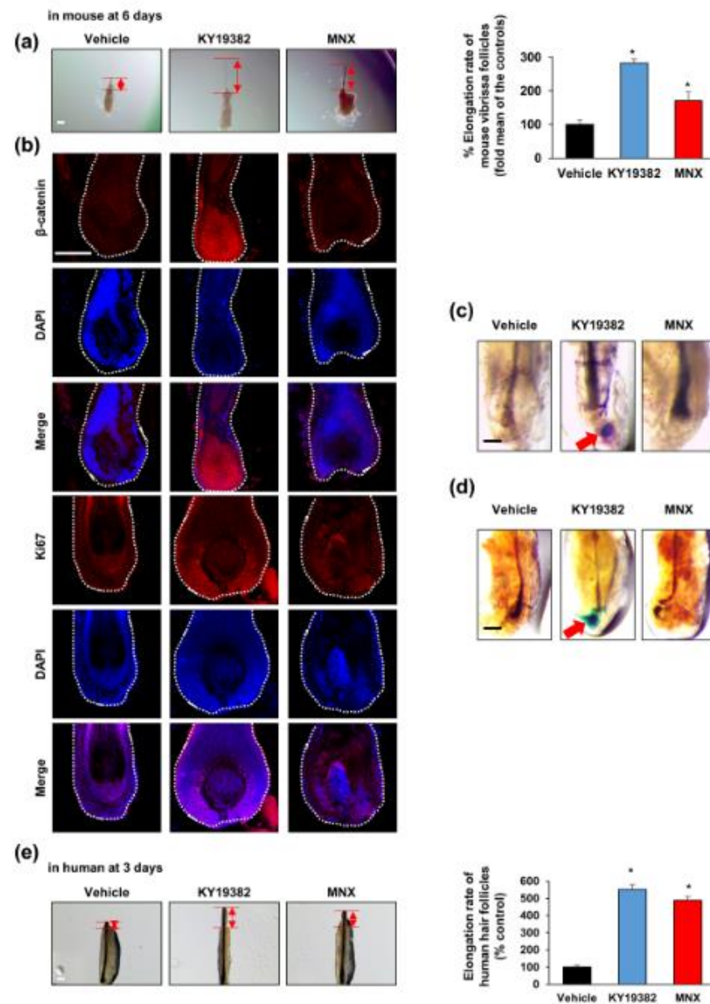

Figure 2. KY19382 accelerates mouse vibrissa and human hair follicle elongation. (a-d) Mouse vibrissa follicles were cultured with vehicle (0.1% DMSO), 5 μM KY19382, or 100 μM MNX for 6 days (n=5). (a) The length of the vibrissa follicles was measured at 6 days after treatment. The elongation rate of mouse vibrissa follicles was evaluated as the difference in the length of vibrissa follicles, wherein the vibrissa follicle length in the control group at day 6 was considered 100%. Scale bars = 200 μm. (b) IHC analyses of hair bulb of mouse vibrissa follicle for Ki67 and β-catenin. Dashed lines mean mouse vibrissa follicles. (c) Mouse vibrissa follicles were subjected to ALP staining. Arrow indicates ALP-positive region. (d) X-gal staining of vibrissa follicles from *Axin2*$^{lacZ/+}$ mice. Arrow indicates X-gal-positive region. (b-d) Scale bars = 100 μm. (e) Human hair follicles were incubated with vehicle (0.1% DMSO), 5 μM KY19382, or 100 μM MNX for 3 days. The length of human hair follicles was evaluated at 3 days after treatment with 5 μM KY19382 or 100 μM MNX. The elongation rate of human follicles was calculated as the difference in the length of human follicles wherein the human follicle length in the control group at day 3 was considered 100% (n=15). Scale bars = 200 μm. Values are expressed as means ± SEM (* P < 0.05).

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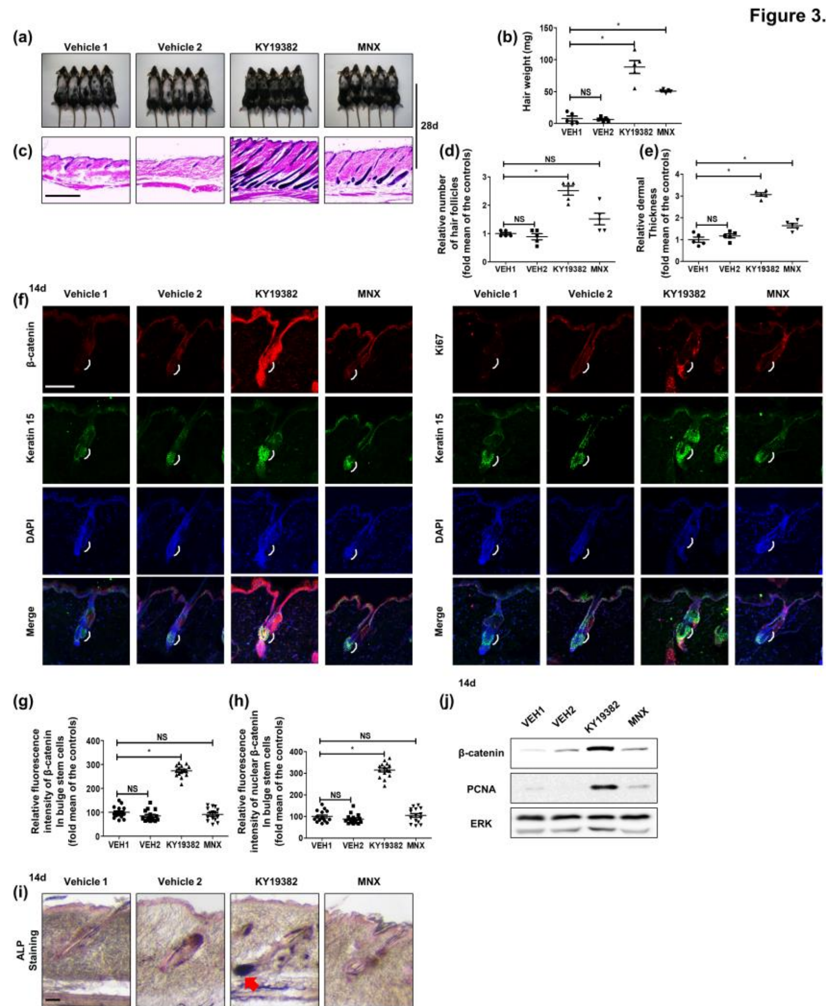

Figure 3. KY19382 stimulates hair regrowth *in vivo*. C57BL/6N mice were treated with vehicle 1 (PEG400), vehicle 2 (50% (v/v) ethanol, 30% water, and 20% propylene glycol), 0.5 mM KY19382 or 100 mM MNX for 14 or 28 days (n=5). (a) The gross image showed hair regrowth in mice treated with each drug for 28 days. (b) Quantitative measurements of the weight of regrown hairs. (c) H&E staining to evaluate the hair follicles of skins with different treatments. (d-e) Quantitative analyses of the relative number of hair follicles and dermal thickness of H&E staining images (n=5). (f) IHC staining for keratin 15, β-catenin, and Ki67 using the dorsal skin of mice treated with each drug for 14 days. Lines show keratin 15-positive bulge stem cells region. (g-h) Quantitative estimations of the total and nuclear β-catenin intensities in bulge stem cells (n=15 taken in 5 different images). (i) ALP staining was performed to evaluate the ALP activity of hair follicles with different treatments. Arrow points to the ALP-positive region. (j) Immunoblotting analyses for β-catenin, PCNA, and ERK. Scale bars = 100 μm. Values are expressed as means ± SEM (* $P < 0.05$; NS, not significant).

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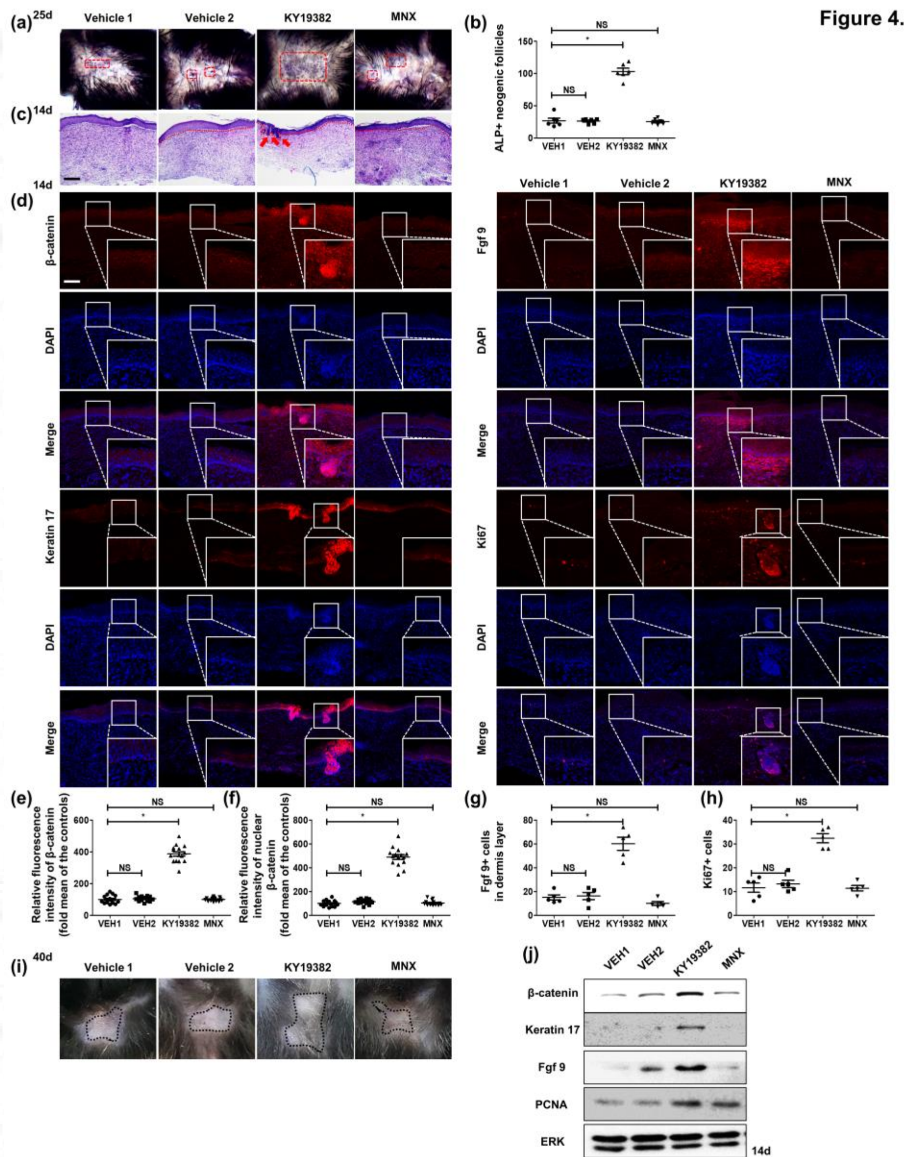

Figure 4. KY19382 stimulates WIHN *in vivo*. C57BL/6N mouse wounds were treated with 0.5 mM KY19382, 100 mM MNX, vehicle 1 (PEG400), or vehicle 2 (50% (v/v) ethanol, 30% water, and 20% propylene glycol) for 14, 25, or 40 days (n=6). (a) ALP staining to evaluate the neogenic follicles of mice treated with each drug for 25 days. (b) Quantitative measurements of ALP-positive neogenic follicles. Dashed lines show ALP-positive neogenic hair follicles. (c) H&E staining to evaluate newly formed hair follicles in mice treated with each drug for 14 days. Dashed lines mean the boundary between the epidermis and dermis. Arrows show newly formed follicles (d) IHC staining for fgf 9, keratin 17, β-catenin and Ki67. (e-f) Quantitative measurement of the nuclear and total β-catenin intensities of neogenic hair follicle cells (n=15 taken in 5 different images). (g) Quantitative analyses of fgf 9-positive dermal cells and (h) Ki67-positive newly formed hair follicle cells (each n=5). (i) Gross images showed newly formed hair in mice after treatment for 40 days. (j) Immunoblotting analyses for fgf 9, keratin 17, β-catenin, PCNA, and ERK for 14 days. Scale bars = 100 µm. Values are expressed as means ± SEM (* $P < 0.05$; NS, not significant).

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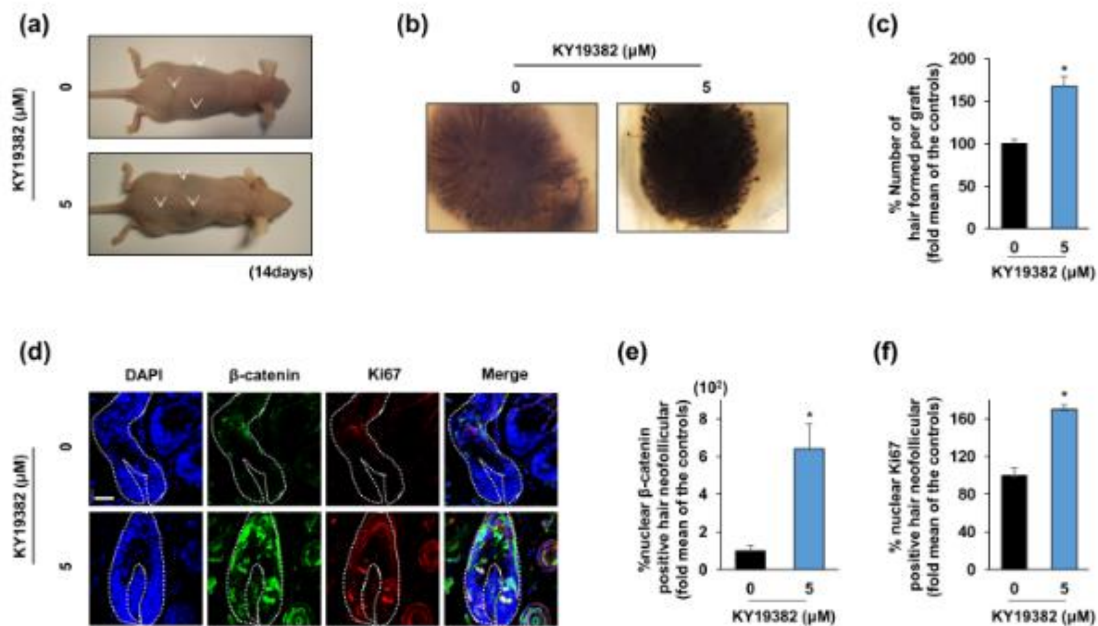

Figure 5. KY19382 induces hair follicle neogenesis in hairless mice. Mouse dermal cells were cultured with vehicle (0.1% DMSO) or 5 μM KY19382 for 72 hours, and then subcutaneously injected with epidermal cells into hairless mice. The hair follicle neogenesis was analyzed at 14 days after transplantation (n=5). (a) Hair follicle neogenesis at the injected area of hairless mice. Arrow indicates the hair regeneration area on the skin. (b) Magnified image of area exhibiting hair follicle neogenesis. (c) Quantitative analyses of regenerated hair follicles. (d) IHC analyses for β-catenin and Ki67. (e-f) Quantitative analyses of the nuclear β-catenin intensity and Ki67-positive cells of regenerated hair follicles. Dashed lines mean hair follicles. Scale bars = 50 μm. Values are expressed as means ± SEM (* $P < 0.05$).

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