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Vanillic Acid Stimulates Anagen Signaling via the PI3K/Akt/β-Catenin Pathway in Dermal Papilla Cells

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Abstract

The hair cycle (anagen, catagen, and telogen) is regulated by the interaction between mesenchymal cells and epithelial cells in the hair follicles. The proliferation of dermal papilla cells (DPCs), mesenchymal-derived fibroblasts, has emerged as a target for the regulation of the hair cycle. Here, we show that vanillic acid, a phenolic acid from wheat bran, promotes the proliferation of DPCs via a PI3K/Akt/Wnt/β-catenin dependent mechanism. Vanillic acid promoted the proliferation of DPCs, accompanied by increased levels of cell-cycle proteins cyclin D1, CDK6, and Cdc2 p34. Vanillic acid also increased the levels of phospho(ser473)-Akt, phospho(ser780)-pRB, and phospho(thr37/46)-4EBP1 in a time-dependent manner. Wortmannin, an inhibitor of the PI3K/Akt pathway, attenuated the vanillic acid-mediated proliferation of DPCs. Vanillic acid-induced progression of the cell-cycle was also suppressed by wortmannin. Moreover, vanillic acid increased the levels of Wnt/β-catenin proteins, such as phospho(ser9)-glycogen synthase kinase-3β, phospho(ser552)-β-catenin, and phospho(ser675)-β-catenin. We found that vanillic acid increased the levels of cyclin D1 and Cox-2, which are target genes of β-catenin, and these changes were inhibited by wortmannin. To investigate whether vanillic acid affects the downregulation of β-catenin by dihydrotestosterone (DHT), implicated in the development of androgenetic alopecia, DPCs were stimulated with DHT in the presence and absence of vanillic acid for 24 h. Western blotting and confocal microscopy analyses showed that the decreased level of β-catenin after the incubation with DHT was reversed by vanillic acid. These results suggest that vanillic acid could stimulate anagen and alleviate hair loss by activating the PI3K/Akt and Wnt/β-catenin pathways in DPCs.

Key Words: Vanillic acid, Dermal papilla cells, Anagen, PI3K/Akt, β-Catenin

INTRODUCTION

Hair loss is a distressing disorder associated with various factors, including hormone imbalance, autoimmune diseases, stress, and chemotherapy. Several known types of hair loss are androgenetic alopecia (AGA), telogen effluvium, alopecia areata, and chemotherapy-induced alopecia (Cotsarelis and Millar, 2001; Hunt and McHale, 2005; Ito, 2010). Minoxidil (Rogain®) and finasteride (Propecia®) have been approved by the Food and Drug Administration (FDA) for treatment of alopecia. Minoxidil was developed as a vasodilator for the treatment of hypertension (Price, 1999). Although the mechanism of action of minoxidil is unclear, this drug stimulates hair growth by activating the Wnt/β-catenin pathway, opening the ATP-sensitive K^+ channels (K_{ATP} channels), upregulating the vascular endothelial growth factor (VEGF), and inhibiting apoptosis in dermal papilla cells (DPCs) (Lachgar *et al*., 1998; Han *et al*., 2004; Shorter *et al*., 2008; Kwack *et al*., 2011).

DPCs are mesenchyme-derived fibroblasts that interact with various types of epithelial cells and communicate with hair germ cells or stem cells in the hair follicles (Stenn and Paus, 2001; Greco *et al*., 2009). DPCs play an important role in hair growth and regeneration, especially in maintaining hair follicles during the growth phase, which requires proliferation of DPCs (Stenn and Paus, 2001; Kwack *et al*., 2011). Cell proliferation is closely related to the progression of the cell-cycle distinguished by the G0/G1, S, and G2/M phases and the changes of the cell-cycle associated protein levels, including

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cyclin, cyclin-dependent kinases (CDKs), and CDK inhibitors (Johnson and Walker, 1999). In particular, it is known that the level of cyclin D1, a target gene of the Wnt/β-catenin pathway, increases with the progression from G0/G1 to S phase during the cell cycle (Prall *et al*., 1997). The phosphoinositide 3-kinase (PI3K)/Akt pathway plays a key role in the proliferation of several types of cells, including cancer cells, keratinocytes, and DPCs (Han *et al*., 2004; Hong *et al*., 2012; Wang *et al*., 2017). Previous studies have shown that minoxidil can prevent apoptosis of DPCs by activating Akt and delay the transition of the hair cycle into the regression phase by activating the Wnt/β-catenin pathway in DPCs (Han *et al*., 2004; Kwack *et al*., 2011). On the other hand, DHT inhibits the Wnt/β-catenin pathway in DPCs, which in part contributes to AGA (Kang *et al*., 2015).

Several studies have focused on the identification of materials that promote hair growth. A previous study has reported the hair growth effects of wheat bran (Kang *et al*., 2013); however, the specific component that promotes hair growth has not been identified. Wheat bran is a source of structurally diverse bioactive compounds, such as phenolic acids, minerals, and polyphenols (Stevenson *et al*., 2012). Vanillic acid, a phenolic acid, is a major component of wheat bran, which has been reported to exhibit anti-oxidative and hepatoprotective activity (Itoh *et al*., 2009; Amin *et al*., 2017). In patients with AGA, methyl vanillate with a structure similar to that of vanillic acid could promote hair growth by activating the Wnt/β-catenin pathway (Tosti *et al*., 2016). However, biological activity of vanillic acid in DPCs has not been investigated. Therefore, we examined whether vanillic acid could stimulate anagen signaling in DPCs by promoting the proliferation of DPCs and activating the PI3K/Akt and Wnt/β-catenin pathways. We also investigated the effects of vanillic acid on the suppression of the Wnt/β-catenin pathway by DHT.

MATERIALS AND METHODS

Reagents

The following reagents were used in this study: dimethyl sulfoxide (DMSO), minoxidil, and propidium iodide (PI) from Sigma-Aldrich (St. Louis, MO, USA); Dulbecco's modified Eagle's medium (DMEM) from Hyclone Inc (Logan, UT, USA); fetal bovine serum (FBS), trypsin-EDTA, and penicillin/streptomycin solution (Pen Strep) from Gibco (Grand Island, NY, USA); water-soluble tetrazolium (WST) assay (EZ-Cytox) from Daeil Lab Service (Seoul, Korea); PRO-PREP protein extraction solution from iNtRON Biotechnology (Seoul); bovine serum albumin (BSA) from Bovogen Biologicals Pty Ltd (Victoria, Australia); anti-phospho(ser9)-GSK-3, anti-phospho(ser552)-β-catenin, anti-phospho(ser675)-β-catenin, anti-phospho(ser780)-pRB, anti-phospho(ser473)-Akt, and anti-phospho(thr37/46)-4EBP1 from Cell Signaling Technology (Danvers, MA, USA); anti-cyclin dependent kinases 6 (CDK6), anti-cyclin E, anti-cdc2 (p34), anti-$p27^{kip1}$, anti-β-catenin, anti-Cox-2, anti-α-tubulin, anti-β-actin, horseradish peroxidase (HRP) labeled anti-mouse IgG, anti-rabbit IgG, and anti-goat IgG secondary antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-Lamin B1 from Abcam (Cambridge, UK); wortmannin, anti-rabbit Alexa Fluors® 594, and anti-mouse Alexa Fluors 488® from Invitrogen (Carlsbad, CA, USA); Vectastain from Vector Laboratories (Burlingame, CA, USA); Dulbecco's phosphate-buffered saline (DPBS) from WelGENE (Daegu, Korea); polyvinylidene fluoride (PVDF) membranes from Bio-Rad (Hercules, CA, USA); X-ray film from Agfa-Gevaert (Mortsel, Belgium); Westar Nova 2.0 from Cyanagen (Bologna, Italy). Vanillic acid was purchased from Sigma-Aldrich and dissolved in DMSO to the final concentration that did not exceed 0.2%.

Cell culture

Rat vibrissa immortalized DPCs were donated by the Skin Research Institute of Amore Pacific R&D Center (Yongin, Korea). DPCs were cultured every three days in an incubator at 37°C and 5% CO_2 atmosphere in DMEM media containing 1% Pen Strep and 10% heat-inactivated FBS.

WST assay

The proliferation of DPCs was measured using the WST kit according to the manufacturer's protocol. DPCs (1.0×10^4 cells/mL) were seeded in a 96-well plate in DMEM medium containing 1% FBS for 24 h and then treated with vanillic acid (0.5, 1, 5, 10 and 50 μg/mL) or minoxidil (10 μM) for 48 h. In some cases, the cells were pre-incubated with wortmannin (10 nM) for 2 h and treated with vanillic acid (10 μg/mL) for 48 h. WST dye (10 μL) was added to the wells in a 5% CO_2 incubator and allowed to react for 3 h. The absorbance was measured at 450 nm using a Versamax microplate reader (Molecular Devices, Sunnyvale, CA, USA). All experiments were repeated three times, the mean absorbance value for each group was obtained, and the results displayed the change compared with the mean absorbance value of the control group.

Cell cycle analysis

DPCs (5.0×10^5 cells/60 mm dish) were incubated in DMEM media containing 1% FBS for 24 h. DPCs were pre-incubated with wortmannin (10 nM) for 2 h and treated with vanillic acid (10 μg/mL) for 24 h. The cells were harvested, washed with PBS, and then fixed with 70% ethanol at –20°C for 30 min. The fixed cells were washed with PBS and incubated with RNase A (50 μg/mL) and PI (50 μg/mL) for 30 min at 37°C. The fluorescence intensity of stained cells was analyzed using FACSCalibur equipped with Cell Quest Software (Becton-Dickinson, San Jose, CA, USA).

Western blotting

DPCs (5.0×10^5 cells/dish) were preincubated with DMEM media containing 1% FBS for 24 h. The cells were treated with vanillic acid (10 μg/mL) for 24 h or 0-120 min. To examine whether vanillic acid affects the PI3K/Akt pathway, the cells were treated with wortmannin (10 nM) in the absence or presence of vanillic acid (10 μg/mL). To investigate whether vanillic acid suppresses the action of DHT, the cells were treated with vanillic acid (10 μg/mL) and then with DHT (100 nM) for 24 h. For protein extraction, the cells were harvested, washed with PBS, and lysed in PRO-PREP protein extraction solution. The cell lysate was centrifuged for 15 min at 21,000×*g* to obtain the supernatant, which was kept at –20°C. The protein concentration was measured by the Bradford method using BSA as a reference material. The cell lysate proteins (10-20 μg) were separated on 8%-12% SDS-PAGE gels and transferred onto PVDF membranes. Blocking of the membranes was performed at room temperature for 2 h in a Tween-20-TBS (T-TBS; 0.1% Tween-20, 50 mM Tris, pH 7.6, 150 mM NaCl) so-

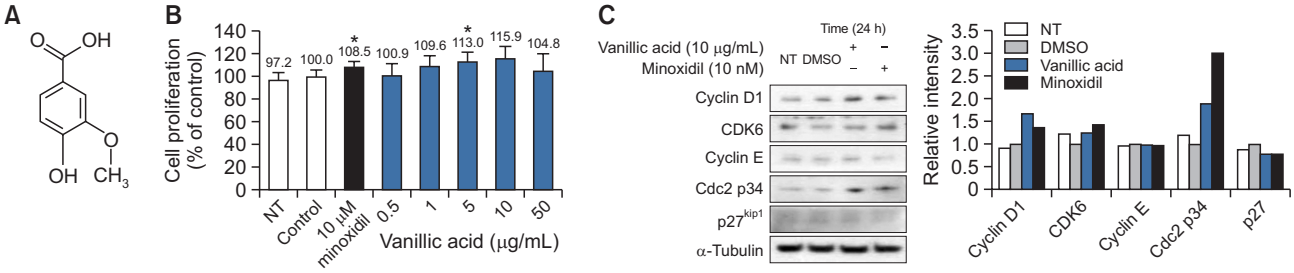

Fig. 1. Vanillic acid promotes the proliferation of dermal papilla cells. (A) Structure of vanillic acid. (B) The viability of the dermal papilla cells (DPCs) was determined by WST assay. The cells were stimulated with various concentrations of vanillic acid or minoxidil for 24 h. The data from three independent experiments are presented as the mean ± SD. **p*<0.05 vs. vehicle-treated control. (C) DPCs were treated with vanillic acid (10 μg/mL) or minoxidil (10 μM) for 24 h, and the protein levels were examined by immunoblotting using specific antibodies. NT, not treated; DMSO, dimethyl sulfoxide.

lution containing 5% nonfat dried milk. The membranes were incubated with specific primary antibodies at 4°C overnight. The membranes were washed five times with T-TBS and incubated with HRP labeled anti-mouse IgG, anti-rabbit IgG, or anti-goat IgG secondary antibodies at 25°C for 1 h. The membranes were incubated in Westar Nova 2.0 ECL solution for 1 min and exposed to X-ray film.

Immunofluorescent staining

DPCs (5.0×10^3 cells/well) were seeded in 8-well chamber slides (Nalgene Nunc International, Rochester, NY, USA) for 24 h prior to be stimulated with vanillic acid (10 μg/mL) or minoxidil (10 μM) for 1 h. The cells were treated with DHT (100 nM) in the absence or presence of vanillic acid (10 μg/mL) for 24 h. For immunofluorescent staining, the cells were washed with cold PBS and fixed with 4% paraformaldehyde for 15 min. The fixed cells were washed with PBS and permeabilized with 0.1% Triton™ X-100 for 15 min. The cells were blocked with a blocking solution (0.1% Tween-20-PBS) containing 1% BSA and 22.52 mg/mL glycine at room temperature for 1 h and incubated with a primary antibody against phospho(ser552)-β-catenin, phospho(ser675)-β-catenin, β-catenin, or α-tubulin at 4°C overnight. The cells were washed with PBS and incubated with a corresponding Alexa Fluor® 488- or Alexa Fluor® 594-conjugated secondary antibody at room temperature for 1 h. The stained cells were mounted in Vectastain (Vector Laboratories) containing DAPI. Images were visualized using a FluoView® FV1200 Confocal Microscope (Olympus, Tokyo, Japan).

Statistical analysis

The experimental data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using SigmaStat Software ver. 3.5 (Systat Software Inc., San Jose, CA, USA). The difference between treated and control groups was determined by the Student's *t*-test. Statistical significance was set at a *p*-value <0.05.

RESULTS

Vanillic acid promotes the proliferation of DPCs

To investigate the effect of vanillic acid on hair follicle cells, we evaluated the proliferation of DPCs following the incubation with vanillic acid for 48 h by WST assay. When DPCs were treated with vanillic acid (0.5, 1, 5, 10, and 50 μg/mL), vanillic acid promoted the proliferation of DPCs compared with the control group (100.0% ± 6.1%) by 100.9% ± 10.8%, 109.6% ± 9.2%, 113.0% ± 8.6% (p<0.05), 115.9% ± 10.7% (p<0.05), and 104.8% ± 1.5%, respectively (Fig. 1B). As shown in Fig. 1B, 10 μM of minoxidil, a positive control, also significantly promoted the proliferation of DPCs by 108.5% ± 5.5% (p<0.05). Cell proliferation is regulated by the progression of the cell cycle, which is accompanied by the changes in cell cycle protein levels of cyclin dependent kinases (CDKs), cyclins, and CDK inhibitors (Whittaker *et al*., 2017). To understand the mechanism of vanillic acid-induced cell proliferation, the levels of cell-cycle proteins, such as cyclin D1, CDK6, cyclin E, Cdc2 p34, and $p27^{kip1}$, were examined 24 h after the vanillic acid treatment. As shown in Fig. 1C, vanillic acid increased the levels of cyclin D1, CDK6, and Cdc2 p34, whereas the levels of cyclin E and $p27^{kip1}$ were not affected. As expected, minoxidil, a positive control, increased the levels of cyclin D1, CDK6, and Cdc2 p34, while the level of $p27^{kip1}$ decreased (Fig. 1C). These results suggest that vanillic acid promotes the proliferation of DPCs by changing the levels of cell-cycle proteins.

Vanillic acid promotes the proliferation of DPCs by activating the PI3K/Akt pathway

The PI3K/Akt pathway plays an important role in the proliferation and survival of various types of cells, and the activation of Akt by minoxidil increases the proliferation of human DPCs (Han *et al*., 2004; Jin *et al*., 2007). To evaluate whether vanillic acid could activate Akt, DPCs were stimulated with vanillic acid (10 μg/mL) for 0-120 min. Vanillic acid increased the levels of phospho(ser473)-Akt after 30-120 min (Fig. 2A). In addition, it was observed that the levels of phospho(ser780)-pRB and phospho(thr37/46)-4EBP1 were increased by vanillic acid (Fig. 2A). To determine whether the vanillic acid-induced proliferation was mediated by activation of the PI3K/Akt pathway, DPCs in the absence or presence of wortmannin (10 nM), an inhibitor of the PI3K/Akt pathway, were treated with vanillic acid (10 μg/mL). As shown in Fig. 2B, the increased proliferation of vanillic acid-treated cells was significantly suppressed in the presence of wortmannin. To monitor the cell cycle changes caused by activating the PI3K/Akt pathway, DPCs were preincubated with wortmannin (10 nM) for 2 h and treated with vanillic acid (10 μg/mL) for 24 h, and the changes of the cell-cycle distribution were analyzed by flow cytometry. As shown in Fig. 2C, the treatment with vanillic acid increased

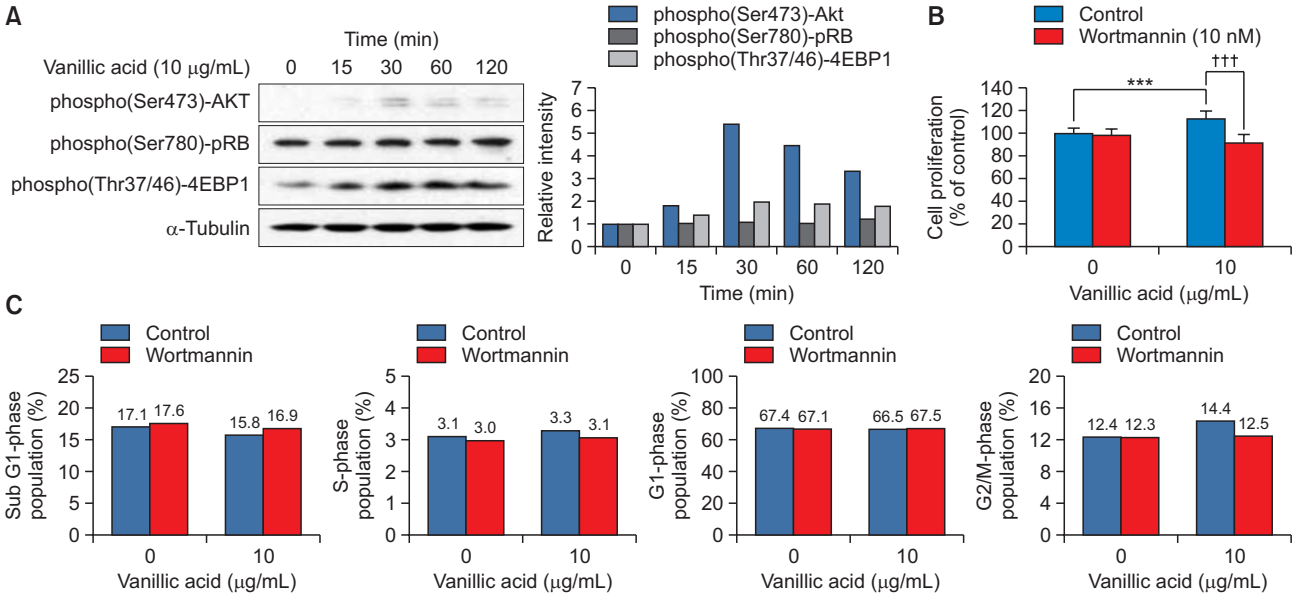

Fig. 2. Vanillic acid promotes the proliferation of DPCs via activation of PI3K/Akt pathway. (A) The effects of vanillic acid on the levels of phospho-Akt, phoshpo-pRB, and phospho-4EBP1. DPCs were treated with vanillic acid (10 μg/mL) for 0-120 min, and the levels of proteins were analyzed by immunoblotting using specific antibodies. (B) The proliferation of DPCs exposed to vanillic acid (10 μg/mL) with or without wortmannin (10 nM) for 48 h was examined by WST assay. (C) Quantitative comparison of cell cycle distribution induced by vanillic acid in the presence or absence of wortmannin for 24 h. ***p<0.001 vs. vehicle-treated control; $^{\dagger\dagger\dagger}p$<0.001 vs. wortmannin-treated group.

the G2/M phase population compared to that of the control. The increase of the G2/M phase population in the vanillic acid-treated cells was suppressed in the presence of wortmannin, suggesting that the activation of the PI3K/Akt pathway by vanillic acid contributes to the proliferation of DPCs.

Vanillic acid activates the Wnt/β-catenin pathway in DPCs

The Wnt/β-catenin pathway, regulated by various factors, such as protein kinase A (PKA), Akt, and glycogen synthase-3β (GSK3β) (Hedgepeth *et al*., 1997; Monick *et al*., 2001; Hino *et al*., 2005), is necessary for the regulation of diverse biological events, including cell proliferation, hair growth, and hair regeneration (Ito *et al*., 2007; Kwack *et al*., 2011). To examine whether vanillic acid activates the Wnt/β-catenin pathway, DPCs were stimulated with vanillic acid (10 μg/mL) for 0-120 min. As shown in Fig. 3A, vanillic acid increased the levels of phospho(ser9)-GSK3β, phospho(ser552)-β-catenin, and phospho(ser675)-β-catenin after 15-120 min. Confocal microscopy data showed that vanillic acid increased the levels of phospho(ser552)-β-catenin and phospho(ser675)-β-catenin in the cytoplasm and nucleus (Fig. 3B, 3C). The phosphorylation of β-catenin by minoxidil was increased similarly to that by the vanillic acid (Fig. 3B, 3C). These results indicate that vanillic acid could increase the levels of active β-catenins via the regulation of GSK3β. GSK3β is a downstream target of PI3K/Akt, which in turn regulates the expression of β-catenin (Monick *et al*., 2001). To evaluate the mechanism underlying the activation of the Wnt/β-catenin pathway by vanillic acid via PI3K/Akt, we examined the levels of phospho(ser675)-β-catenin, β-catenin, and Cox-2, a target gene of β-catenin, following a 24 h treatment with vanillic acid in the presence or absence of wortmannin. As shown in Fig. 3D, vanillic acid increased the levels of phospho(ser675)-β-catenin, β-catenin, Cox-2, and cyclin D1, and the increased levels of these proteins were attenuated by wortmannin (Fig. 3D). These results suggest that the activation of the Akt pathway by vanillic acid contributes to the activation of the Wnt/β-catenin pathway.

Vanillic acid restores DHT-downregulated β-catenin level in DPCs

Dihydrotestosterone, implicated in the development of AGA, suppresses the cell-cycle progression and Wnt/β-catenin pathway in DPCs (Kang *et al*., 2015). To determine whether vanillic acid could restore the downregulation of β-catenin by DHT, DPCs were treated with DHT (100 nM) in the absence or presence of vanillic acid (10 μg/mL) for 24 h. The decreased levels of β-catenin and Cox-2, a target gene of β-catenin, observed in the DHT treated cells were restored by vanillic acid (Fig. 4A). Confocal microscopy data showed that DHT decreased the level of β-catenin, but pretreatment with vanillic acid attenuated the DHT-induced decrease of β-catenin level (Fig. 4B). These results suggest that vanillic acid can restore the DHT-induced downregulation of the Wnt/β-catenin pathway.

DISCUSSION

Previous *in vitro* and *in vivo* research has demonstrated the promotion of hair growth by wheat bran. Here we demonstrate that vanillic acid from wheat bran increases the proliferation of DPCs via activation of the Wnt/β-catenin and PI3K/Akt pathways.

Although the mechanism of hair growth is still unclear, the proliferation of DPCs has been associated with hair growth. The shape of the dermal papilla changes throughout the hair-cycle, affected by the changes in the number of cells within the dermal papilla (Elliott *et al*., 1999; Stenn and Paus, 2001). In addition, because both the cell cycle proteins and cell cycle

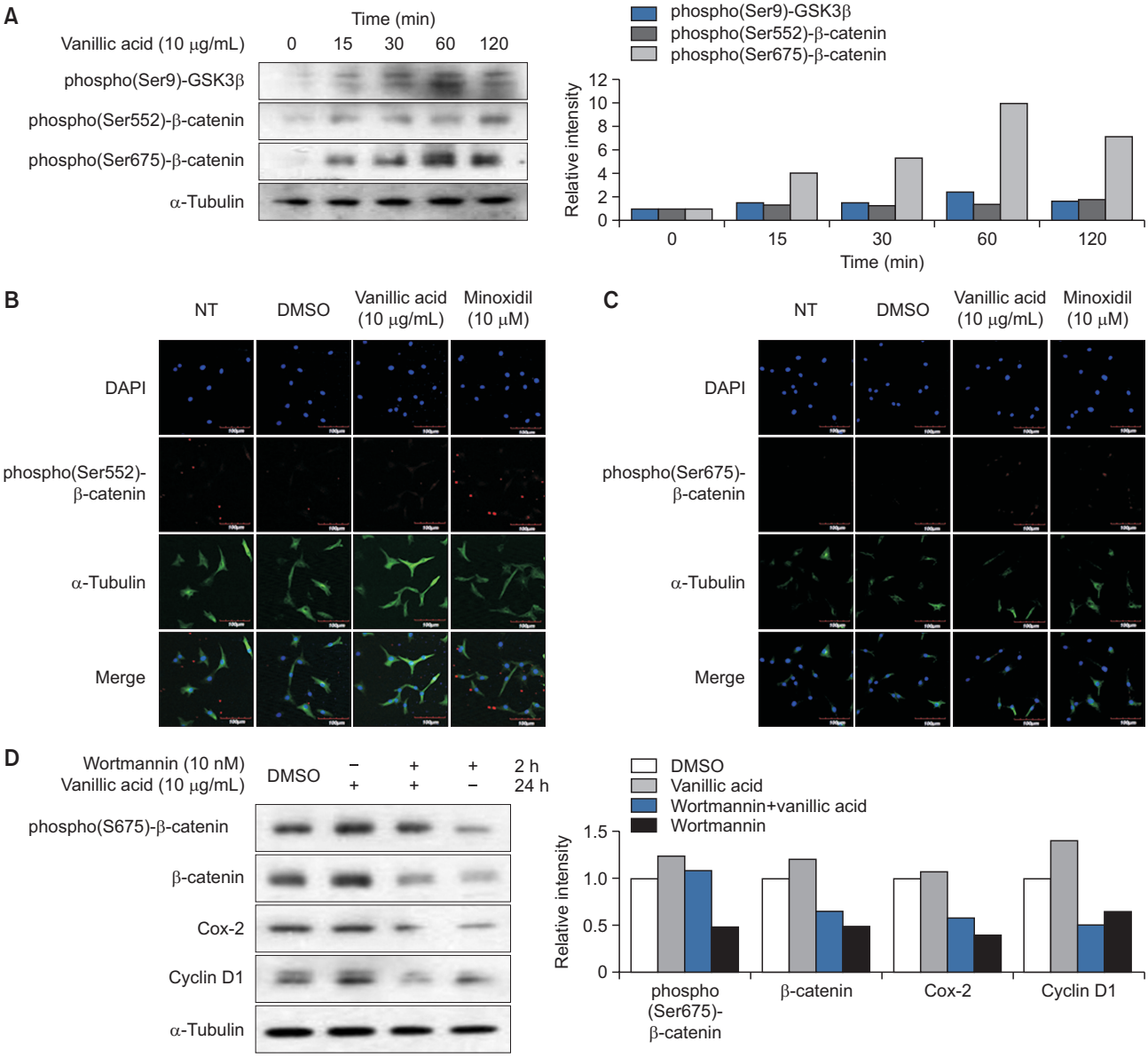

Fig. 3. Vanillic acid activates the Wnt/β-catenin pathway in DPCs. (A) The effects of vanillic acid on the levels of Wnt/β-catenin proteins. DPCs were stimulated with vanillic acid (10 μg/mL) for 0-120 min. The cell lysate from DPCs was subjected to immunoblotting using specific antibodies. (B, C) The effects of vanillic acid on the localization of phospho(ser552)-β-catenin and phospho(ser675)-β-catenin. Cellular localization of phospho(ser552)-β-catenin and phospho(ser675)-β-catenin was analyzed using confocal microscopy. Scale bars 100 μm in B and C. (D) The effects of vanillic acid on the levels of phospho(ser675)-β-catenin, β-catenin, Cox-2, and cyclin D1. DPCs with or without wortmannin (10 nM) were treated with vanillic acid (10 μg/mL) for 24 h, and the protein levels were examined by immunoblotting. NT, not treated; DMSO, dimethyl sulfoxide.

itself alter the cell proliferation in diverse types of cells (Prall *et al*., 1997; Hong *et al*., 2012), it is likely that the regulation of cell cycle proteins can increase the proliferation of DPCs in the hair follicles. Cyclin D1 and CDK6 induce the progression of cell cycle via the phosphorylation of pRB in the G1 phase, while Cdc2 p34 promotes the transition of G2 to M phase (Meyerson and Harlow, 1994; Johnson and Walker, 1999; Tashiro *et al*., 2007). As shown in Fig. 1 and Fig. 2A, vanillic acid significantly increased the proliferation of DPCs and the levels of cell cycle proteins (cyclin D1, CDK6, Cdc2 p34, and phospho-pRB). Our results indicate that vanillic acid promotes the proliferation of DPCs by altering the cell cycle proteins, including cyclin D1, CDK6, Cdc2, p34, and phospho(ser780)-pRB. Other changes such as the progression of the cell cycle and protein synthesis are also associated with DPC proliferation, and the protein 4EBP1 is required for the translation of proteins necessary for cell cycle progression (Kang *et al*., 2015; Lian *et al*., 2017). In this study, the increased G2/M phase population and up-regulation of phospho(thr37/46)-4EBP1 were observed in the vanillic acid-treated cells (Fig. 2A), indicating that the vanillic acid-induced proliferation of DPCs is regulated by manipulating the level of phospho(thr37/46)-4EBP1 and cell cycle pro-

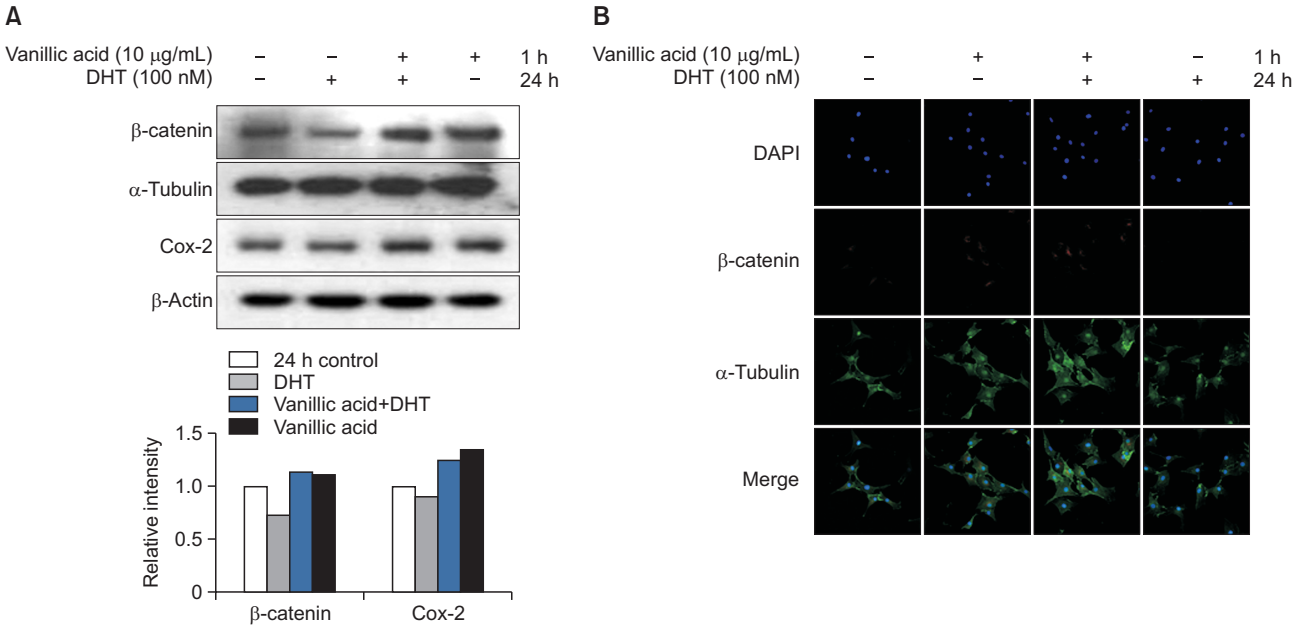

Fig. 4. Vanillic acid restores DHT-downregulated β-catenin level in DPCs. DPCs were treated with DHT (100 nM) in the presence or absence of vanillic acid (10 μg/mL) for 24 h. (A) The effects of vanillic acid on the regulation of the Wnt/β-catenin pathway by DHT. The levels of β-catenin and Cox-2 were analyzed by immunoblotting. (B) The localization of β-catenin was analyzed by confocal microscopy (magnification of 400×).

gression.

The mechanism of DPC proliferation promoted by vanillic acid may involve the activation of the PI3K/Akt and/or Wnt/β-catenin pathways. A previous study has found that cell proliferation is associated with GSK3β, a modulator of the Wnt/β-catenin pathway, regulated by the PI3K/Akt pathway (Jin *et al*., 2007). Akt, a serine/threonine kinase, is a direct downstream target of PI3K, and it is crucial to various aspects of cell proliferation, survival, and apoptosis (Franke *et al*., 2003). In our study, the phosphorylation/activation of Akt by vanillic acid increased the proliferation of DPCs via the upregulation of cell-cycle proteins (Fig. 1, 2). The Wnt/β-catenin pathway is regulated by various factors; in particular, PKA induces the phosphorylation of β-catenin at ser552 and ser675, and the activation of Akt induces the phosphorylation of β-catenin at ser552 and phosphorylation of GSK3β at ser9, eventually activating the Wnt/β-catenin pathway (Monick *et al*., 2001; Hino *et al*., 2005). In this study, vanillic acid induced the activation of the Wnt/β-catenin proteins following the activation of the PI3K/Akt pathway (Fig. 2, 3). The activation of Wnt/β-catenin resulted in the stabilization of β-catenin in the cytoplasm and increased the translocation of β-catenin into the nucleus, thereby, controlling the level of target genes, such as cyclin D1 and cox-2 (Hedgepeth *et al*., 1997; Monick *et al*., 2001; Kang *et al*., 2015). The vanillic acid-mediated activation of β-catenin was also confirmed by the increased β-catenin translocation into the nucleus (Fig. 3B, 3C). As shown in Fig. 3D, vanillic acid also upregulated the levels of Cox-2 and cyclin D1, which are the target genes of β-catenin, and the level of β-catenin. These changes were attenuated by wortmannin, indicating that the vanillic acid-mediated proliferation in DPCs is regulated by the PI3K/Akt/Wnt/β-catenin pathway. Consistent with this notion, minoxidil prolongs the duration of the anagen phase, possibly, due to the activation of the Wnt/β-catenin pathway by altering the PKA and Akt pathways in human DPCs (Kwack *et al*., 2011). Therefore, based on the above mechanism, vanillic acid could activate the anagen phase and promote hair growth.

DHT, a potent androgen, plays a crucial role in the pathogenesis of AGA (Sinclair, 1998). Our previous study partially supports the DHT-mediated AGA development, demonstrating that DHT can attenuate the cell cycle progression by inhibiting translocation of β-catenin into the nucleus in DPCs (Kang *et al*., 2015). In this study, we demonstrated that vanillic acid exhibits a protective effect on the downregulation of β-catenin by DHT (Fig. 4). In addition, we investigated the effects of vanillic acid on the levels of growth factors, the opening of K_{ATP} channels, and the inhibition of the TGF-β pathway related to the regulation of the hair growth (Guo *et al*., 1996; Suzuki *et al*., 2000; Yano *et al*., 2001; Soma *et al*., 2002; Shorter *et al*., 2008). However, we observed that vanillic acid does not affect the levels of VEGF, FGF-7, and FGF-10 mRNA, the opening of K_{ATP} channels, or the phosphorylation of smad2/3, a mediator of the TGF-β pathway (data not shown).

In conclusion, we evaluated the effects of vanillic acid on the proliferation of DPCs and verified that vanillic acid selectively regulates the PI3K/Akt/Wnt/β-catenin pathway. These findings suggest that vanillic acid could stimulate the anagen phase by activating the PI3K/Akt/Wnt/β-catenin pathway and potentially alleviate hair loss.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

Amin, F. U., Shah, S. A. and Kim, M. O. (2017) Vanillic acid attenuates Aβ 1-42-induced oxidative stress and cognitive impairment in mice. *Sci. Rep.* **7**, 40753.

Cotsarelis, G. and Millar, S. E. (2001) Towards a molecular understanding of hair loss and its treatment. *Trends Mol. Med.* **7**, 293-301.

Elliott, K., Messenger, A. G. and Stephenson, T. J. (1999) Differences in hair follicle dermal papilla volume are due to extracellular matrix volume and cell number: implications for the control of hair follicle size and androgen responses. *J. Invest. Dermatol.* **113**, 873-877.

Franke, T. F., Hornik, C. P., Segev, L., Shostak, G. A. and Sugimoto, C. (2003) PI3K/Akt and apoptosis: size matters. *Oncogene* **22**, 8983.

Greco, V., Chen, T., Rendl, M., Schober, M., Pasolli, H. A., Stokes, N., Dela Cruz-Racelis, J. and Fuchs, E. (2009) A two-step mechanism for stem cell activation during hair regeneration. *Cell Stem Cell* **4**, 155-169.

Guo, L., Degenstein, L. and Fuchs, E. (1996) Keratinocyte growth factor is required for hair development but not for wound healing. *Genes Dev.* **10**, 165-175.

Han, J. H., Kwon, O. S., Chung, J. H., Cho, K. H., Eun, H. C. and Kim, K. H. (2004) Effect of minoxidil on proliferation and apoptosis in dermal papilla cells of human hair follicle. *J. Dermatol. Sci.* **34**, 91-98.

Hedgepeth, C. M., Conrad, L. J., Zhang, J., Huang, H. C., Lee, V. M. and Klein, P. S. (1997) Activation of the Wnt signaling pathway: a molecular mechanism for lithium action. *Dev. Biol.* **185**, 82-91.

Hino, S., Tanji, C., Nakayama, K. I. and Kikuchi, A. (2005) Phosphorylation of beta-catenin by cyclic AMP-dependent protein kinase stabilizes beta-catenin through inhibition of its ubiquitination. *Mol. Cell. Biol.* **25**, 9063-9072.

Hong, J. Y., Boo, H. J., Kang, J. I., Kim, M. K., Yoo, E. S., Hyun, J. W., Koh, Y. S., Kim, G. Y., Maeng, Y. H., Hyun, C. L., Chang, W. Y., Kim, Y. H., Kim, Y. R. and Kang, H. K. (2012) (1S,2S,3E,7E,11E)-3,7,11,15-Cembratetraen-17,2-olide, a cembrenolide diterpene from soft coral Lobophytum sp., inhibits growth and induces apoptosis in human colon cancer cells through reactive oxygen species generation. *Biol. Pharm. Bull.* **35**, 1054-1063.

Hunt, N. and McHale, S. (2005) The psychological impact of alopecia. *BMJ* **331**, 951-953.

Ito, M., Yang, Z., Andl, T., Cui, C., Kim, N., Millar, S. E. and Cotsarelis, G. (2007) Wnt-dependent de novo hair follicle regeneration in adult mouse skin after wounding. *Nature* **447**, 316-320.

Ito, T. (2010) Hair follicle is a target of stress hormone and autoimmune reactions. *J. Dermatol. Sci.* **60**, 67-73.

Itoh, A., Isoda, K., Kondoh, M., Kawase, M., Kobayashi, M., Tamesada, M. and Yagi, K. (2009) Hepatoprotective effect of syringic acid and vanillic acid on concanavalin a-induced liver injury. *Biol. Pharm. Bull.* **32**, 1215-1219.

Jin, S., Pang, R. P., Shen, J. N., Huang, G., Wang, J. and Zhou, J. G. (2007) Grifolin induces apoptosis via inhibition of PI3K/AKT signalling pathway in human osteosarcoma cells. *Apoptosis* **12**, 1317-1326.

Johnson, D. G. and Walker, C. L. (1999) Cyclins and cell cycle checkpoints. *Annu. Rev. Pharmacol. Toxicol.* **39**, 295-312.

Kang, J. I., Moon, J., Kim, E. J., Lee, Y. K., Koh, Y. S., Yoo, E. S., Kang, H. K. and Yim, D. (2013) The hair growth effects of wheat bran. Korean. *J. Pharmacogn.* **44**, 384-390.

Kang, J. I., Kim, S. C., Kim, M. K., Boo, H. J., Kim, E. J., Im, G. J., Kim, Y. H., Hyun, J. W., Kang, J. H., Koh, Y. S., Park, D. B., Yoo, E. S. and Kang, H. K. (2015) Effects of dihydrotestosterone on rat dermal papilla cells *in vitro*. *Eur. J. Pharmacol.* **757**, 74-83.

Kwack, M. H., Kang, B. M., Kim, M. K., Kim, J. C. and Sung, Y. K. (2011) Minoxidil activates beta-catenin pathway in human dermal papilla cells: a possible explanation for its anagen prolongation effect. *J. Dermatol. Sci.* **62**, 154-159.

Lachgar, S., Charveron, M., Gall, Y. and Bonafe, J. (1998) Minoxidil upregulates the expression of vascular endothelial growth factor in human hair dermal papilla cells. *Br. J. Dermatol.* **138**, 407-411.

Lian, X., Zhao, J., Wu, X., Zhang, Y., Li, Q., Lin, S., Bai, X. Y. and Chen, X. (2017) The changes in glucose metabolism and cell proliferation in the kidneys of polycystic kidney disease mini-pig models. *Biochem. Biophys. Res. Commun.* **488**, 374-381.

Meyerson, M. and Harlow, E. (1994) Identification of G1 kinase activity for cdk6, a novel cyclin D partner. *Mol. Cell. Biol.* **14**, 2077-2086.

Monick, M. M., Carter, A. B., Robeff, P. K., Flaherty, D. M., Peterson, M. W. and Hunninghake, G. W. (2001) Lipopolysaccharide activates Akt in human alveolar macrophages resulting in nuclear accumulation and transcriptional activity of beta-catenin. *J. Immunol.* **166**, 4713-4720.

Prall, O. W., Sarcevic, B., Musgrove, E. A., Watts, C. K. and Sutherland, R. L. (1997) Estrogen-induced activation of Cdk4 and Cdk2 during G1-S phase progression is accompanied by increased cyclin D1 expression and decreased cyclin-dependent kinase inhibitor association with cyclin E-Cdk2. *J. Biol. Chem.* **272**, 10882-10894.

Price, V. H. (1999) Treatment of hair loss. *N. Engl. J. Med.* **341**, 964-973.

Shorter, K., Farjo, N. P., Picksley, S. M. and Randall, V. A. (2008) Human hair follicles contain two forms of ATP-sensitive potassium channels, only one of which is sensitive to minoxidil. *FASEB J.* **22**, 1725-1736.

Sinclair, R. (1998) Male pattern androgenetic alopecia. *BMJ* **317**, 865-869.

Soma, T., Tsuji, Y. and Hibino, T. (2002) Involvement of transforming growth factor-beta2 in catagen induction during the human hair cycle. *J. Invest. Dermatol.* **118**, 993-997.

Stenn, K. S. and Paus, R. (2001) Controls of hair follicle cycling. *Physiol. Rev.* **81**, 449-494.

Stevenson, L., Phillips, F., O'sullivan, K. and Walton, J. (2012) Wheat bran: its composition and benefits to health, a European perspective. *Int. J. Food Sci. Nutr.* **63**, 1001-1013.

Suzuki, K., Yamanishi, K., Mori, O., Kamikawa, M., Andersen, B., Kato, S., Toyoda, T. and Yamada, G. (2000) Defective terminal differentiation and hypoplasia of the epidermis in mice lacking the Fgf10 gene. *FEBS Lett.* **481**, 53-56.

Tashiro, E., Tsuchiya, A. and Imoto, M. (2007) Functions of cyclin D1 as an oncogene and regulation of cyclin D1 expression. *Cancer Sci.* **98**, 629-635.

Tosti, A., Zaiac, M. N., Canazza, A., Sanchis-Gomar, F., Pareja-Galeano, H., Alis, R., Lucia, A. and Emanuele, E. (2016) Topical application of the Wnt/beta-catenin activator methyl vanillate increases hair count and hair mass index in women with androgenetic alopecia. *J. Cosmet. Dermatol.* **15**, 469-474.

Wang, R., Zhao, Z., Zheng, L., Xing, X., Ba, W., Zhang, J., Huang, M., Zhu, W., Liu, B., Meng, X., Bai, J., Li, C. and Li, H. (2017) MicroRNA-520a suppresses the proliferation and mitosis of HaCaT cells by inactivating protein kinase B. *Exp. Ther. Med.* **14**, 6207-6212.

Whittaker, S. R., Mallinger, A., Workman, P. and Clarke, P. A. (2017) Inhibitors of cyclin-dependent kinases as cancer therapeutics. *Pharmacol. Ther.* **173**, 83-105.

Yano, K., Brown, L. F. and Detmar, M. (2001) Control of hair growth and follicle size by VEGF-mediated angiogenesis. *J. Clin. Invest.* **107**, 409-417.