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Wnt/β-catenin signaling activator restores hair regeneration suppressed by diabetes mellitus

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Diabetes mellitus is one of the most prevalent diseases in modern society. Many complicationssuch as hepatic cirrhosis, neuropathy, cardiac infarction, and so on are associated with diabetes. Although a relationship between diabetes and hair loss has been recently reported, the treatment of diabetic hair loss by Wnt/β-catenin activators has not been achieved yet. In this study, we found that the depilation-induced anagen phase was delayed in both *db*/*db* mice and high-fat diet (HFD) and streptozotocin (STZ)-induced diabetic mice. In diabetic mice, both hair regrowth and wound-induced hair follicle neogenesis (WIHN) were reduced because of suppression of Wnt/β-catenin signaling and decreased proliferation of hair follicle cells. We identified that KY19382, a small molecule that activates Wnt/β-catenin signaling, restored the capabilities of regrowth and WIHN in diabetic mice. The Wnt/β-catenin signaling activator also increased the length of the human hair follicle which was decreased under high glucose culture conditions. Overall, the diabetic condition reduced both hair regrowth and regeneration with suppression of the Wnt/β-catenin signaling pathway. Consequently, the usage of Wnt/β-catenin signaling activators could be a potential strategy to treat diabetes-induced alopecia patients. [BMB Reports 2022; 55(11): 559-564]

INTRODUCTION

Diabetes mellitus is a metabolic disorder associated with abnormally high blood glucose levels. The number of patients with diabetes has been increasing worldwide (1). There are two types of diabetes: type 1 and type 2. Type 1 diabetes is caused by an autoimmune disease that destroys pancreatic β cells and reduces insulin secretion, whereas type 2 diabetes is more common and attributed to the dysfunction of pancreatic β cells and insulin resistance, leading to reduced responses to insulin (2, 3). Both types of diabetes result in glucose accumulation in the blood. Hyperglycemia adversely affects many organs. There are various complications associated with diabetes, including retinopathy, nephropathy, and neuropathy (4). These complications of diabetes are related to the Wnt/β-catenin signaling pathway. For instance, diabetic retinopathy is caused by up-regulation of Wnt/β-catenin signaling (5), whereas diabetic foot ulcer and diabetes-related Alzheimer's diseases are caused by down-regulation of Wnt/β-catenin signaling (6, 7). A recent study has shown that diabetes can inhibit hair regrowth by inhibiting Wnt/β-catenin signaling (8). However, this study was mainly focused on the effect of diabetes on hair follicle cycle transition, not on hair follicle neogenesis itself, using a hair depilation model. These findings suggested that Wnt/β-catenin signaling is a desirable target for the treatment of diabetic hair loss. However, drugs targeting Wnt/β-catenin signaling to treat diabetic hair loss are not currently available.

The number of patients with alopecia has been increasing. Alopecia occurs in men, women, and even young people (9). It is characterized by gradual shrinkage of hair follicles and thinning of the hair shaft. These processes are related to Wnt/β-catenin signaling pathway (10). When cells are not stimulated by Wnt ligands, β-catenin is phosphorylated by the destruction complex, subsequently ubiquitinated and then degraded (11). However, when the Wnt/β-catenin signaling is activated by Wnt ligands, the β-catenin is no longer degraded and enters the nucleus to act as a transcription factor, resulting in the expression of its target genes (12). Activated Wnt/β-catenin pathway plays many roles in the morphogenesis, growth, and regeneration of hair follicles (13). In particular, wound-induced hair follicle neogenesis (WIHN), a model for mammalian hair regeneration, is strongly associated with Wnt/β-catenin signaling (14). Moreover, alkaline phosphatase (ALP), which plays an essential role in anagen induction and WIHN, is increased by Wnt/β-catenin signaling (15). Therefore, Wnt/β-catenin signaling, which plays a significant role in hair maintenance, is

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reduced in alopecia patients (16). Some studies have investigated the relationship between hair loss and obesity (17). However, the mechanism involved in alopecia of diabetes patients has not been clarified yet.

In this study, to investigate the role of diabetes in hair loss, we determine changes in hair formation of HFD and STZ-induced and *db*/*db* diabetic model mice by evaluating new hair follicle formation and hair induction. Both regrowth and neogenesis of hair were decreased in diabetic mice, and those were caused by a decrease in Wnt/β-catenin signaling with the suppression of proliferation and ALP in diabetic mice. To further characterize the role of Wnt/β-catenin signaling in hair growth, we tested effects of KY19382 known to promote both regrowth and neogenesis of hair by activating Wnt/β-catenin signaling via inhibition of CXXC5 and GSK-3β functions (18). Wnt/β-catenin signaling activator induced hair induction and regeneration in diabetic mice. In addition, KY19382 increased lengths of human hair follicles that were decreased under high glucose culture conditions. Taken together, these results suggest that diabetes mellitus can lead to hair loss by decreasing Wnt/β-catenin signaling. The Wnt/β-catenin signaling activator could improve hair elongation and neogenesis in diabetes conditions by increasing proliferation and ALP expression. Overall, Wnt/β-catenin signaling activators could be used for promoting hair growth in diabetes-induced alopecia patients.

RESULTS

Diabetic mice show delay of depilation-induced hair cycle

To study hair promotion in diabetic mice, we used type 2 diabetes mouse models induced by HFD feeding and STZ administration and leptin receptor deficiency (*db*/*db*) (Supplementary Fig. 1A, B). To investigate the alteration of hair cycle in the diabetic mouse model, we performed depilation to induce a synchronized hair cycle (19). The color of mouse dorsal skins and hematoxylin and eosin (H&E) staining confirmed a delayed plucking-induced hair cycle in diabetic mice (Supplementary Fig. 2A and 3A-C). Quantitative analysis of the number of hair follicles and dermal thickness, indicators of hair regrowth, also showed that hair regrowth was delayed in HFD and STZ-induced diabetic mice (Supplementary Fig. 2B, C). When estimating the hair cycle stage of each hair follicle, there was a difference in the hair cycle stage between normal, and HFD and STZ-induced diabetic mice on each day (Supplementary Fig. 2D).

β-Catenin expression increases during the anagen phase, and gradually decreases during the progression of the hair cycle toward catagen phases (20). We confirmed that β-catenin was belatedly activated as shown by immunohistochemical (IHC) analysis of diabetic mice (Supplementary Figs. 3D and 4A). Expression profiles of total and nuclear β-catenin were retarded in diabetic mice (Supplementary Fig. 4B, C). The expression of AE13, a differentiation marker, was also delayed in the hair cycle (Supplementary Fig. 3D and 4A). In summary, we found that depilation-induced progression to the anagen phase was delayed in diabetic mice. We also confirmed that diabetic mice, compared to normal mice, slowly expressed β-catenin, which is essential for anagen induction.

Induction of hair cycle and neogenic follicles is diminished in diabetic mice attributed to reduced Wnt/β-catenin signaling and proliferation

To identify changes in Wnt/β-catenin signaling and proliferation in depilated normal and diabetic mice, we examined normal and diabetic mice at 5 or 7 days after plucking (Supplementary Fig. 1C). Fewer hair follicles progressed toward the anagen phase in diabetic mice than in normal mice as shown by H&E staining of mouse dorsal skins at 5 days after plucking (Fig. 1A, B). Expression levels of β-catenin and proliferation markers, PCNA and Ki67, were decreased in keratin 15-positive hair stem cells (21) of diabetic mice (Fig. 1C and Supplementary Fig. 5B). Real-time qPCR showed that expression levels of the Wnt/β-catenin signaling target genes, *Axin2* and *Wisp1*, were reduced in diabetic mice (Supplementary Fig. 5C). ALP staining for dorsal skins of mice at 7 days post plucking revealed that diabetic mice reduced their hair induction capacity (Fig. 1D).

To confirm the effect on WIHN in diabetic mice, we generated 2.25 cm^2 wounds in normal and diabetic mice (Supplementary Fig. 1C). Whole-mount ALP staining of skin tissues showed that ALP-positive follicles were significantly reduced

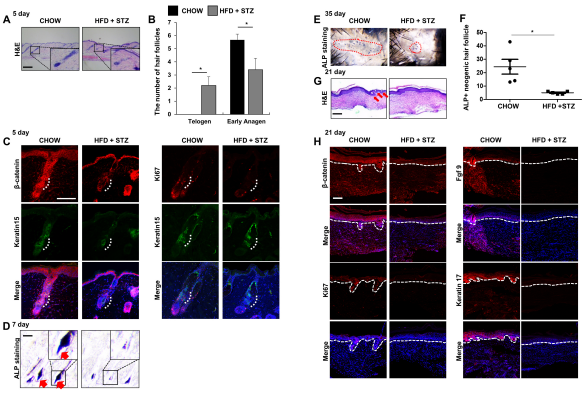

Fig. 1. Alteration of hair growth in HFD and STZ-induced diabetic mice. (A-D) Mouse dorsal skins were harvested at 5 or 7 days after depilation. (A) H&E staining of mouse dorsal skins at 5 day after depilation. (B) Quantitative analysis of each hair cycle stage. (C) IHC staining for keratin 15, Ki67, and β-catenin. (D) ALP staining at 7 days after depilation. (E-H) Mouse wounds were harvested at 21 or 35 days after wounding. (E) ALP staining to determine newly formed follicles at 35 days after wounding. (F) Quantitative calculation of neogenic hair follicles. (G) H&E staining showed neogenic hair follicles at 21 days after wounding. (H) IHC staining for keratin 17, fgf 9, β-catenin, and Ki67 in wound regions. Scale bars: 100 μm for A, C, D, G, and H. n = 5 mice per group for B and F. Values are expressed as means ± SEM. *P < 0.05.

in diabetic mice compared to those in normal mice (Fig. 1E, F and Supplementary Fig. 3E, F). H&E staining showed that the number of neogenic hair follicles was diminished in diabetic mice at 21 days after wound induction (Fig. 1G). Consistently, Wnt/β-catenin signaling and proliferation markers were reduced in diabetic mice (Fig. 1H and Supplementary Figs. 3G, 5D). Expression levels of Fgf 9 and Keratin 17, as neogenic follicle markers (20, 22, 23), were also decreased in diabetic mice (Fig. 1H and Supplementary Fig. 3G). Furthermore, mRNA levels of *Axin2* and *Wisp1* were diminished in diabetic mice (Supplementary Fig. 5E). Overall, diabetic mice showed decreased capabilities of hair induction and neogenesis due to reduction of Wnt/β-catenin signaling.

Wnt/β-catenin signaling activator attenuates anagen induction in HFD and STZ-induced diabetic mice

Previously, we have reported that KY19382 promoted regrowth and regeneration of hair via activation of Wnt/β-catenin signaling (18). To determine whether a Wnt/β-catenin signaling activator could recover the reduced hair induction capability in diabetic mice, we applied KY19382 or minoxidil (MNX), a FDA-approved topical medication for alopecia (24), to dorsal skins of plucked mice (Supplementary Fig. 1D). After 9 days of daily application, onlymice applied with KY19382 showed improved anagen induction capability (Fig. 2A). Enhanced progression of telogen to anagen phase transition in KY19382-treated mice was confirmed by H&E staining (Fig. 2B, C). IHC staining showed that KY19382 increased expression levels of β-catenin and proliferation markers in the bulge area where stem cells are located (Fig. 2D and Supplementary Fig. 6A). Quantitative analyses also confirmed that KY19382 restored diabetes-induced suppression of proliferation and Wnt/β-catenin signaling (Fig. 2E, F and Supplementary Fig. 6B, C). Moreover, an increase in the ALP signal was correlated with the recovery of hair induction capability in diabetic mice induced by KY19382 treatment (Fig. 2G). Taken together, these data showed that the Wnt/β-catenin signaling activator alleviated hair regrowth in diabetic mice by activating Wnt/β-catenin signaling.

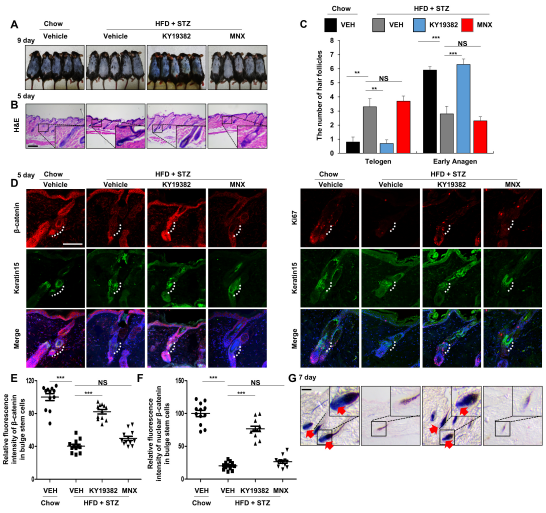

Fig. 2. Effects of Wnt activator on hair cycle in HFD and STZ-induced diabetic mice. After plucking, mouse dorsal skins were treated with 1.5 mM KY19382 or 150 mM MNX for 5, 7 or 9 days. (A) Gross image showing the color of mouse backs at 9 days after plucking. (B) H&E staining at 5 day after treatment. (C) Quantitative calculations of each hair cycle stage. (D) IHC staining for keratin 15, β-catenin and Ki67 of dorsal skins at 5 days after treatment. (E, F) Quantitative evaluations for fluorescence intensities of total and nuclear β-catenin in bulge stem cells. (G) ALP staining of dorsal skins at 7 days. Scale bars: 100 μm for B, D, and G. n = 10 mice per group for C, n = 12 per group for E and F. Values are expressed as means ± SEM. **P < 0.005, ***P < 0.0005, NS, not significant.

Wnt/β-catenin signaling activator ameliorates WIHN in HFD and STZ-induced diabetic mice

To confirm the effect of the Wnt/β-catenin signaling activator on reduced WIHN competence in diabetic mice, we applied KY19382 or MNX onto wounds (Supplementary Fig. 1E). The

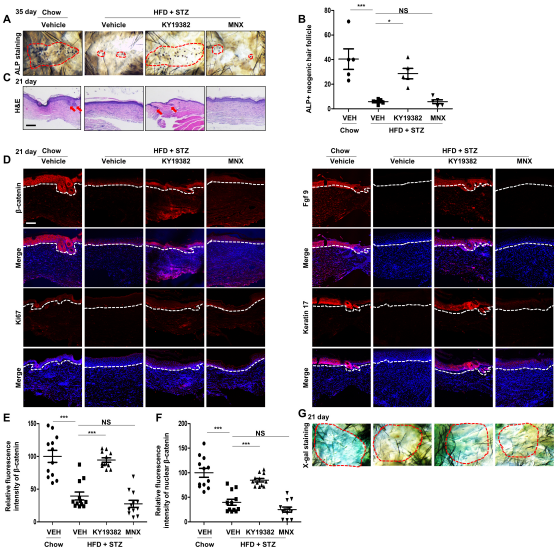

Fig. 3. Influences of Wnt activator on WIHN in HFD and STZ-induced diabetic mice. Mouse wounds were treated with 1.5 mM KY19382 or 150 mM MNX for 21 or 35 days. (A) ALP staining to identify newly formed follicles of mice treated with each compound for 35 days. Dashed lines mean neogenic hair follicles. (B) Quantitative evaluation of ALP-positive newly formed follicles. (C) H&E staining to identify neogenic hair follicles in mice treated with each compound for 21 days and the relative number of neogenic hair follicles. Arrows indicate neogenic hair follicles. (D) IHC staining for fgf 9, keratin 17, β-catenin, and Ki67. Dashed lines indicate a boundary between epidermal and dermal regions. (E, F) Quantitative calculations for fluorescence intensities of total and nuclear β-catenin in neogenic hair follicle cells. (G) X-gal staining of mouse wounds from *Axin2*$^{lacZ/+}$ mice. Scale bars: 100 μm for C and D. n = 5 mice per group for B, n = 12 per group for E, F. Values are expressed as means ± SEM. *P < 0.05, ***P < 0.0005, NS, not significant.

KY19382-applied group, but not the MNX-applied group, showed recovery of neogenic follicles decreased by diabetes (Fig. 3A, B). H&E staining indicated that KY19382 ameliorated the development of neogenic follicles in diabetic mice at 21 days after application (Fig. 3C). Expression levels of β-catenin and proliferation markers in neogenic follicles were restored in KY19382-treated diabetic mice (Fig. 3D-F and Supplementary Fig. 6D, E). Moreover, different from vehicle- and MNX-treated groups, expression levels of the neogenic hair follicle markers were restored in KY19382-treated diabetic mice (Fig. 3D and Supplementary Fig. 6F). Restorative activation of the Wnt/β-catenin signaling by KY19382 treatment was confirmed using Wnt/β-catenin signaling reporter *Axin2*$^{lacZ/+}$ mouse (Fig. 3G). Collectively, these data suggested that the reduced induction of neogenic hair follicles in diabetic mice was recovered by Wnt/β-catenin signaling activator increasing Wnt/β-catenin signaling and cell proliferation.

Wnt/β-catenin signaling activator improves human hair follicle elongation reduced by high glucose conditions

To investigate the effect of a diabetic environment on human hair follicles, we utilized a hyperglycemia *in vitro* model under high glucose conditions (25). We incubated human hair follicles in control or high glucose medium with vehicle, MNX, or KY19382 for 2 or 6 days. After 6 days of growing human hair follicles in the high glucose medium, hair shaft elongation was suppressed. However, KY19382, but not MNX, restored elongation of the hair shaft (Fig. 4A, B). Specific restoration of expression levels of β-catenin and proliferation markers by KY19382 was confirmed by IHC analyses (Fig. 4C-E and Supplementary Fig. 6G, H). Overall, the Wnt/β-catenin signaling activator restored elongation of the human hair follicle suppressed by a high glucose environment.

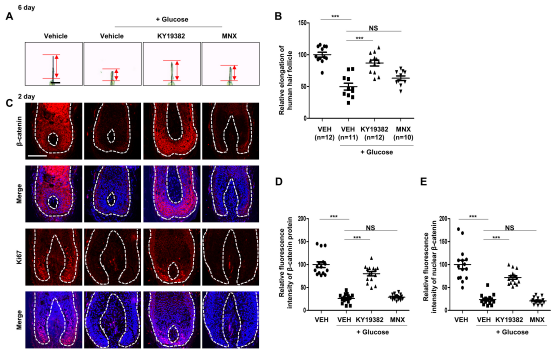

Fig. 4. Effects of Wnt activator on lengths of human hair follicles in a hyperglycemia model. Human hair follicles were cultured in control or high glucose (100 mM) medium with 1 μM KY19382 or 150 μM MNX for 2 or 6 days. (A, B) Hair shaft elongation of human follicles was measured for 6 days. (C) IHC analysis of human hair follicles for β-catenin and Ki67 for 2 days. (D, E) Quantitative measurements for nuclear and total β-catenin in human hair follicle cells. Scale bars: 100 μm for A, C. n = 10-12 follicles per group for B and n = 15 per group for D, E. Values are expressed as means ± SEM. *P < 0.05, ***P < 0.0005, NS, not significant.

DISCUSSION

Diabetes mellitus is one of the major metabolic diseases. Finding an effective treatment for diabetes is an increasingly challenging healthcare issue. Diabetes mellitus is a chronic disease with blood sugar level not adequately controlled (1). Uncontrolled blood sugar level results from dysfunction of insulin production and/or reduced insulin action in tissues (2). Long-term high blood sugar level in diabetic patients is related to malfunctioning of many organs, which might lead to complications (4). Some complications of diabetes are associated with down-regulation of the Wnt/β-catenin signaling pathway. Suppression of Wnt/β-catenin signaling in diabetic patients induces diabetic foot ulcer and Alzheimer's disease (6, 7). Therefore, Wnt activators can be used as therapeutics to improve these complications of diabetes (7, 26).

The number of patients with alopecia has increased significantly. Interest in hair loss treatments is also increasingly (9). Various factors deactivate hair follicle cells, and this inactivation causes miniaturized hair follicles, leading to hair loss (10). Although alopecia patients still possess keratin 15-positive stem cells, these stem cells are inactive (27, 28). Stem cell activation is an important treatment method for hair loss patients primarily through activation of Wnt/β-catenin signaling (29). In this study, we confirmed hair regrowth in diabetic mice treated with a Wnt/β-catenin signaling activator because stem cells were still present in diabetic mice with only inactivation of stem cells (Fig. 3 and Supplementary Fig. 1B). On the contrary, MNX had no effects on diabetic mice as p-ERK levels were not obviously changed in diabetic mice compared to those in mice on a chow diet (Supplementary Fig. 3A); MNX is known to stimulate hair regrowth through p-ERK activation (30). In particular, as MNX is not effective for hair regeneration (18), unlike the Wnt/β-catenin signaling activator, MNX was not effective for WIHN of diabetic mice (Fig. 4). Collectively, these findings suggest that Wnt activators, but not commercially available drugs such as MNX, could be important for treating alopecia induced by diabetes through activation of hair follicle stem cells.

In this study, we found that KY19382 induced hair follicle regeneration in diabetic wounds with a low regenerative capacity. Previous studies have demonstrated that Wnt or Shh activation results in the formation of fully regenerated hair follicles in wounds (31). Herein, we confirmed that KY19382 induced the formation of dermal condensates or dermal papilla in diabetic wounds through ALP staining, although the formation of fully regenerated follicles by KY19382 in diabetic wounds should be further confirmed. Overall, considering the regenerative effect of KY19382 and impaired tissue regeneration ability in diabetic patients, KY19382 can be utilized to induce regeneration of various tissues including hair appendages in diabetic patients.

Hair loss is associated with several chronic diseases such as Lupus disease, syphilis, autoimmune disease, and thyroid disease (32-35). Changes in immunity of chronic diseases result in alteration of the Wnt/β-catenin signaling pathway (36, 37). Alopecia areata, one of the autoimmune diseases, shows reduced Wnt/β-catenin signaling that causes hair loss (16). Furthermore, there have been case studies of increased hair loss in diabetic patients (38) and reports of the hair growth inhibition by inactivated Wnt/β-catenin signaling in diabetes (8). Thus, our findings together with the importance of regulating the Wnt/β-catenin signaling pathway in several chronic diseases suggest that Wnt activators might be potential therapeutic agents for patients with several chronic diseases, including diabetic alopecia.

MATERIALS AND METHODS

All research design and methods were described in the supplemental material.

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CONFLICTS OF INTEREST

K.Y.C is the CEO of CK Regeon Inc. (Seoul, Korea), which has a license to develop and use the compounds disclosed in the publication. The authors have no conflicting interests.

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