Effects of *Hura crepitans* and Its Active Ingredient, Daphne Factor F3, on Dihydrotestosterone-Induced Neurotrophin-4 Activation and Hair Retardation

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Neurotrophin (NT)-4 is known to be an inducer of catagen in the hair cycle, but little is known of its role in the pathogenesis of androgenetic alopecia (AGA). We previously studied the gene expression of dermal papilla cells from AGA patients and controls and found that NT-4 was up-regulated in the AGA patients. In the present study, the etiological relationship between NT-4 and androgen, which is one of the causes of AGA, and the effect of an NT-4 inhibitor on hair growth were investigated. We established a NT-4 luciferase reporter assay system using a roughly 2-kb region upstream of the NT-4 transcriptional start site and investigated an accelerating effect of androgen on NT-4 transcription. We also screened for a NT-4 inhibitor by using the NT-4 reporter assay and evaluated the effects of NT-4 inhibitors on hair growth by using dihydrotestosterone (DHT)-implanted mice. The results show that transcriptional activity of NT-4 was accelerated by androgen, and extract of *Hura crepitans* L. inhibited the DHT-induced NT-4 transcriptional activation and ameliorated the retardation of hair regrowth by DHT-implanted mice. We also isolated the active ingredient in *H. crepitans* and found its structure to be that of 6,7-epoxy-5-hydroxyresiniferonol-14-(2,4-tetradecadienoate), *i.e.*, daphne factor F3. These findings demonstrated that NT-4 activity accelerated by androgen might contribute to the pathogenesis of AGA and indicated that NT-4 inhibitors such as *H. crepitans* and daphne factor F3 might have a salutary effect on AGA.

Key words *Hura crepitans*; daphne factor F3; neurotrophin-4; androgenetic alopecia

Hair follicles exhibit regular cycles of regeneration, known as the hair cycle. This cycle involves phases of growth (anagen), regression (catagen) and rest (telogen).[1,2] Hair follicles consist primarily of epithelial and mesenchymal components and the hair cycle phase is determined by the balance of many stimulatory and inhibitory factors secreted when these components interact.[3,4] Androgenetic alopecia (AGA), also known as male pattern baldness, is a result of shortening of the anagen phase and miniaturization of the hair follicles.[5] Androgen, which is one of the causes of AGA,[6] enters the follicles *via* the blood vessels, binds to androgen receptors (ARs) in the dermal papilla (DP) cells and stimulates or represses the secretion of many growth factors from DP cells. Androgen is reported to stimulate the release of some of these growth factors, such as insulin-like growth factor (IGF)-1,[7] transforming growth factor (TGF)-β1,[8] TGF-β2,[9] and dickkopf (DKK)-1[10] in order to inhibit hair growth.

On the other hand, it has been reported that the neurotrophins: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3 and NT-4 contribute to the pathogenesis of alopecia.[11] It is known that they control the proliferation, differentiation and functional maintenance of nerve cells[12] and are expressed not only in the nervous system but also in the skin, liver and testis. Botchkarev and his co-workers' research has suggested i) that BDNF, NT-3 and NT-4 are expressed from late anagen to catagen in hair follicles of murine skin,[13,14] ii) that all four of these neurotrophins accelerate the development of catagen in murine skin organ cultures,[15] iii) that BDNF or NT-4 knockout mice show significant catagen retardation,[13] iv) that all four neurotrophin proteins bind to the p75kD neurotrophin receptor (p75NTR), which is a member of the tumor necrosis factor (TNF) receptor-related superfamily mediating apoptosis, and v) that none of these four neurotrophins stimulate spontaneous catagen development in organ cultures p75NTR null skin.[16] In addition, exposure to sonic stress inhibits the growth of a hair shaft by inducing catagen prematurely[17,18] and up-regulating NGF expression in hair follicles in mice.[19] The stress-induced premature onset of catagen was inhibited by treatment with an antibody neutralizing NGF.

We previously studied the gene expression of DP cells from AGA patients and individuals without AGA by using a DNA array technique and found that NT-4 mRNA expression was up-regulated in AGA patients.[20] In the present study we analyzed the etiological relationship between NT-4 and androgen to determine whether or not NT-4 plays a role in causing of AGA and searched for a NT-4 inhibitor having an effect on hair growth.

MATERIALS AND METHODS

Plasmid Construction and Luciferase Reporter Assay The upstream region from −1979 to −45 bp of the human NT-4 gene transcriptional start site was amplified by polymerase chain reaction (PCR) and cloned into pCR-Blunt II-TOPO (Invitrogen, CA, U.S.A.). The primers used for PCR were 5′-TCCCCCTCTTTCTTTTGGTCTC-3′ and 5′-GAAGGGGTTGTTCAGGGATTTG-3′. The NT-4 upstream region was excised as a 1890-bp fragment with *Kpn*I digestion and

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was subcloned into the *Kpn*I site of pGL-3 basic Vector (Promega, WI, U.S.A.). Then we constructed the NT-4 Luc plasmid and used it in an NT-4 luciferase reporter assay.

For the luciferase reporter assay, HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) without phenol red (Sigma-Aldrich, MO, U.S.A.) but supplemented with 10% fetal bovine serum (FBS) (Biowest, France) stripped with charcoal (Sigma).[21] The cells were seeded in 24-well plates and cotransfected with 0.24 μg of NT-4 Luc plasmid as a reporter, 0.06 μg of AR expression vector (provided by Prof. S. Kato, University of Tokyo) and 20 ng of pRL-SV40 (Promega) as an internal standard at 70—80% confluence using FuGENE6 transfection reagent (Roche, Basel, Switzerland) following the manufacturer's instructions. The transfectants were cultured for 48 h in the absence or presence of dihydrotestosterone (DHT: 1, 10 or 100 nM) (Wako Pure Chemical Industries, Osaka, Japan). In the evaluation of NT-4 inhibitory activity the transfectants were cultured for 24 h in the presence of 100 nM DHT and then transferred to a medium including 100 nM DHT and a plant extract (at the concentrations indicated in the figures) and incubated for another 24 h. After this 48-h incubation the cells were assayed using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Hair Regrowth Assay Hair regrowth assays were performed as described previously.[22] Male C57BL/6 mice (n=6) were purchased from Nihon SLC (Shizuoka, Japan) and maintained according to a protocol approved by the Animal Care and Use Committee of LION Corporation. The dorsal hair of male mice in the telogen stage (52 d postpartum) was removed with electric hair clippers and shavers, and the anagen stage was induced. Thirty milligrams of DHT was implanted subcutaneously in the neck region of the shaved back. Sham surgery was performed on another group. The next day we started treating the mice with 100 μL of NT-4 inhibitors dissolved in 50% ethanol at the concentrations indicated in the figures and continued the treatments (once daily) for 40 d on the shaved area. Control-group mice were treated with 100 μL of vehicle (50% ethanol) instead of the NT-4 inhibitor solution. Regrown hair could be seen as dark on the pinkish-white shaved skin, and the percentage of the shaved area that became dark was measured. The hair regrowth from day 1 to day 40 after DHT implantation was evaluated using the following scale: 0, 0% (no hair growth); 1, $<$20%; 2, 20—39%; 3, 40—59%; 4, 60—79%; 5, 80—100%.

Plant Materials *Hura crepitans* L. from Peru and *Wikstroemia retusa* W. Grey from Okinawa were purchased from Toyotama Koryo Co. (Tokyo, Japan), and *H. crepitans* from Brazil was purchased from Takasago International Corp. (Tokyo, Japan).

Plant Extraction Dry bark of each plant was ground using a grinder mill, and 50 g of the ground product was extracted in 1 L of 70% ethanol. The extract was concentrated with a rotary evaporator under reduced pressure at 42°C, suspended in distilled water and partitioned with ethyl acetate to obtain ethyl acetate-soluble material. The ethyl acetate-soluble material was evaporated. As a result, we got 0.50 g of *H. crepitans* (from Peru) extract, 0.45 g of *H. crepitans* (from Brazil) extract and 3.60 g of *W. retusa* extract.

Isolation and Identification of the Active Ingredient Included in *H. crepitans* One kilogram of *H. crepitans* from Peru was extracted in 6 L of 70% ethanol by using an accelerated solvent extractor (ASE-200, Dionex, CA, U.S.A.). The extract (80 g) was suspended in water and partitioned with ethyl acetate. The ethyl acetate extract (25 g) was applied to a SiO_2 chromatography column and eluted with isooctane to yield fraction I, with isooctane and diethyl ether at v/v gradations from 19 : 1 to 19 : 4 to yield fractions II—V, with diethyl ether to yield fraction VI, with acetone to yield fraction VII, and with methanol to yield fraction VIII. Fraction VI (3 g) was dissolved in methanol and separated by reversed-phase HPLC (octadecylsilyl) to yield fraction VI-B (0.4 g). Fraction VI-B was further separated by reversed-phase HPLC (octasilyl) to yield fraction VI-B-2 (15 mg).

The chemical structure of VI-B-2 was identified by several methods, including Fourier-transform infrared (FT-IR) spectroscopy (Spectrum 100 FT-IR, Perkin Elmer, MA, U.S.A.), MS (Waters, MA, U.S.A.) and NMR (JEOL, Tokyo, Japan) (the details of the fractionation and structural analyses are being prepared for submission).

Measurement of the Relative Daphne Factor F3 Content The relative daphne factor F3 content in each extract was measured by LC/MS (Micromass ZQ, Waters). The extract was separated by reversed-phase HPLC (octasilyl). MS was performed in the selected ion monitoring (SIM) mode using target ions at $[M+H]^+$ m/z 603.3 and $[M+Na]^+$ m/z 625.3 (molecular mass of daphne factor F3 is 602.3).

RESULTS

Androgen and Androgen Receptor (AR) Complex Directly Activates the Transcription of NT-4 To determine whether or not the upregulation of NT-4 mRNA expression in the DP cells from AGA patients was the result of androgen, we established the NT-4 luciferase reporter assay system using the 1890-b region upstream of the NT-4 transcriptional start site. As shown in Fig. 1, the relative luciferase activity was increased significantly by the addition of 1—100 nM DHT, the activated form of androgen. This result suggests that the upstream region of the NT-4 transcriptional start site has AR binding sites and that AR activated by DHT directly upregulates the transcriptional activity of NT-4.

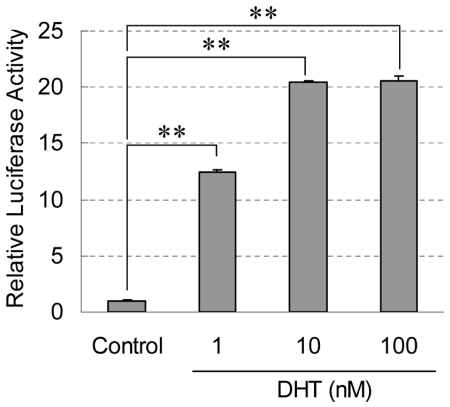

Fig. 1. Activation of NT-4 Promoter by DHT

Luciferase expression from NT-4 promoter reporter constructs in the presence and absence (Control) of DHT. Approximately 2-kb of the region upstream of the NT-4 transcriptional start site was cloned into a luciferase reporter vector. Relative luciferase activity normalized to the activity of a cotransfected internal standard is shown (n=3, mean±S.E.M.). Statistical significance was evaluated by ANOVA with Dunnett's multiple comparison test. **p<0.01.

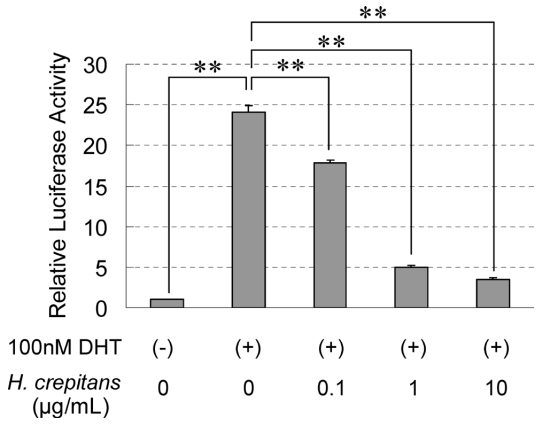

Fig. 2. Effect of *Hura crepitans* on DHT-Induced Transcriptional Activation of NT-4

Luciferase expression from NT-4 promoter reporter constructs in the presence and absence of DHT and *H. crepitans* extract. Each bar shows the mean of triplicates and the error bars show the SEM. Statistical significance was evaluated by ANOVA with Dunnett's multiple comparison test. $^{**}p<0.01$.

***Hura crepitans* L. Inhibits the NT-4 Activation Induced by DHT and Stimulates the Hair Growth Effect** To screen for an agent therapeutic for AGA, we evaluated the NT-4 inhibitory activity of about three hundred plants which our laboratory possessed. Using the NT-4 reporter assay, we found that the extract of *H. crepitans* from Peru significantly inhibited the NT-4 transcriptional activation induced by DHT (Fig. 2). We then evaluated the effect of *H. crepitans* on hair growth by using C57BL/6 mice with DHT implanted under the back skin. We previously reported that DHT implantation remarkably suppressed hair regrowth after depilation.[22] As shown in Fig. 3, DHT-implanted mice treated with vehicle (Control-group) showed a remarkably retardation of hair regrowth. DHT-implanted mice treated with 1 wt% extract of *H. crepitans*, on the other hand, showed an amelioration of the retardation of hair regrowth.[23] This result suggests that *H. crepitans* which inhibits the NT-4 transcriptional activation induced by DHT inhibits the retardation of hair regrowth by DHT.

Daphne Factor F3 Included in *H. crepitans* Contributes to the NT-4 Inhibitory Activity To identify the active ingredient contributing to the effect of *H. crepitans* on hair growth, we separated the *H. crepitans* extract by column chromatography and HPLC and evaluated the NT-4 inhibitory activity of these fractions by using the NT-4 reporter assay. We refined the fraction which showed the highest NT-4 inhibitory activity into a single component, and analyzed its chemical structure by measuring its FT-IR, MS and NMR spectra. The active ingredient was determined to be 6,7-epoxy-5-hydroxyresiniferonol-14-(2,4-tetradecadienoate) (Fig. 4), *i.e.*, daphne factor F3, otherwise known as prohuratoxin[24] or wikstroelide M.[25]

Daphne Factor F3 Ameliorates the Retardation of Hair Regrowth in DHT-Implanted Mice To verify that daphne factor F3 has an effect on hair growth, we treated the back skin of the DHT-implanted mice with 0.01 wt% daphne factor F3. Because pure daphne factor F3 was not available commercially or from other laboratories, we refined daphne factor F3 from *H. crepitans* and used it for the *in vivo* assay. As shown in Fig. 3, DHT-implanted mice treated with 0.01 wt% daphne

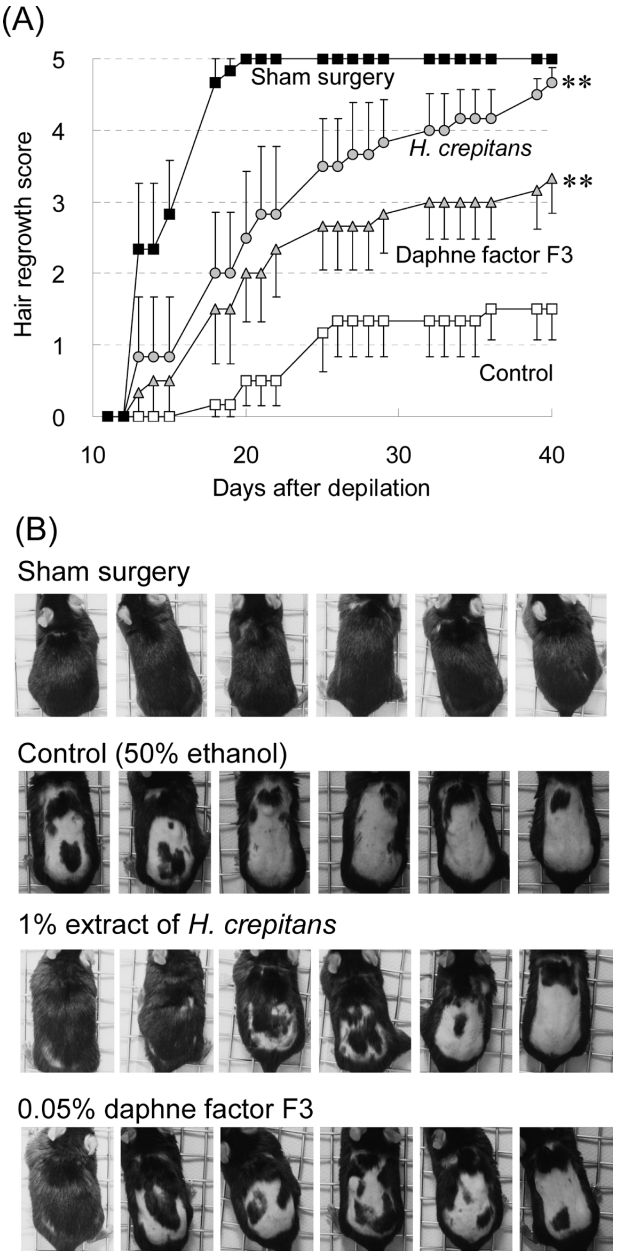

Fig. 3. Effects of *Hura crepitans* and Daphne Factor F3 on Hair Regrowth of DHT-Implanted Mice

(A) Changes in hair regrowth score after depilation. Sham surgery mice (■) and DHT-implanted mice treated with vehicle (Control, □), 1% extract of *H. crepitans* (●) and 0.01% daphne factor F3 (▲). Each point represents the mean for six mice and the error bars show the S.E.M. Data were analyzed by a two-way ANOVA followed by Dunnett's *post hoc* test *versus* the control group at day 40 after depilation. $^{**}p<0.01$. (B) The appearance of hair regrowth at day 26 after depilation.

factor F3 showed less retardation of hair regrowth. This result suggests that daphne factor F3 affects hair growth and is the main ingredient responsible for the activity of *H. crepitans*.

We additionally evaluated the hair growth effect of *Wikstroemia retusa* A. Grey, which also includes daphne factor F3,[26] and of *H. crepitans* from Brazil. One can see in Fig. 5 that *W. retusa*, unlike *H. crepitans* from Peru, did not affect hair regrowth. The effect of *H. crepitans* from Brazil was the same as that of *W. retusa* (data not shown).

To find a cause for the different levels of the hair regrowth effects of these three extracts, we measured the relative daphne factor F3 content in each extract by LC/MS. As shown in

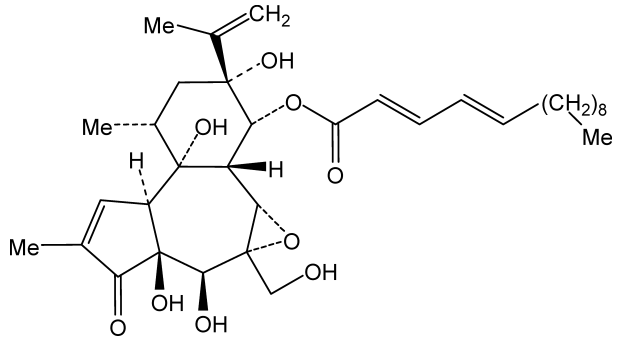

Fig. 4. Chemical Structure of Daphne Factor F3

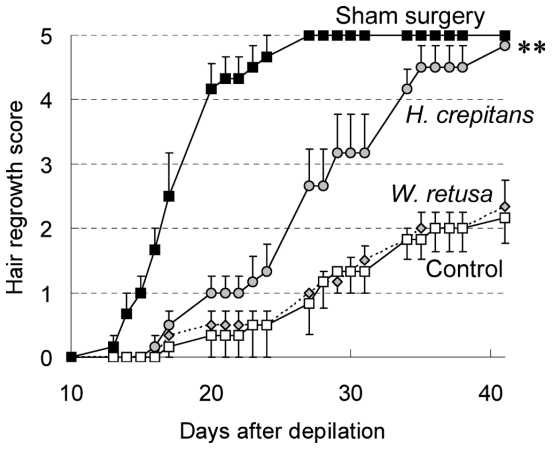

Fig. 5. Changes in Hair Regrowth Score Resulting from Treatment with *Wikstroemia retusa* and *Hura crepitans* (from Peru) after Depilation

Sham surgery mice (■) and DHT-implanted mice treated with vehicle (Control, □), 1% extract of *H. crepitans* (from Peru, ●) and 1% extract of *W. retusa* (◆). Each point represents the mean for six mice and the error bars show the S.E.M. Data were analyzed by a two-way ANOVA followed by Dunnett's *post hoc* test *versus* the control group at day 41 after depilation. $^{**}p<0.01$.

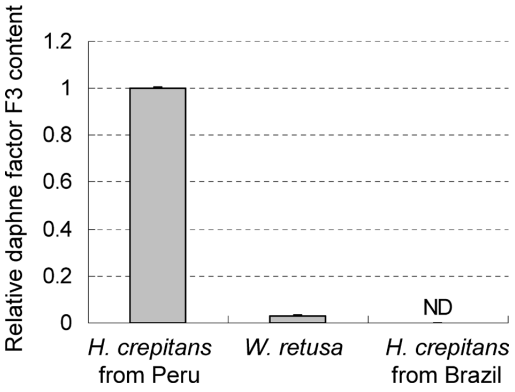

Fig. 6. Relative Daphne Factor F3 Content in *Hura crepitans* from Peru and Brazil and *Wikstroemia retusa*

The relative daphne factor F3 content in each extract was measured by LC/MS. The content in *W. retusa* was 3% of that in *H. crepitans* from Peru, and daphne factor F3 was not detectable in *H. crepitans* from Brazil.

Fig. 6, the relative daphne factor F3 contents of in *W. retusa* and *H. crepitans* from Brazil were less than that in *H. crepitans* from Peru (the contents in *H. crepitans* from Peru: 1.00, *W. retusa*: 0.03, *H. crepitans* from Brazil: below detectable limit). These results suggest that extracts that include large amounts of daphne factor F3 show the hair growth effect.

DISCUSSION

NT-4 is known to induce catagen and accelerate hair loss by binding to the p75NTR mediating apoptosis.[13—16] In this study we found that NT-4 was directory up-regulated by the androgen and androgen receptor (AR) complex. This result supports the idea that NT-4 plays an important role in the pathogenesis of AGA. On binding of androgen and AR, AR undergoes a conformational change and recruits coactivator complexes for transactivation through direct DNA binding to androgen response elements (AREs) in AR target gene promoters.[27,28] Upstream of the NT-4 transcriptional start site here are some regions similar to AREs, and some of these regions could be the AR target upregulating the transcriptional activity of NT-4. To clarify the AR binding site in the NT-4 promoter region, an NT-4 reporter assay using deletion mutants is indispensable.

Our results show that *H. crepitans* from Peru inhibited NT-4 transcription accelerated by DHT and ameliorated the retardation of hair regrowth by DHT. *H. crepitans* is euphorbiaceous and in Amazonis has since ancient times been used in traditional folk therapy for Hansen's disease and syphilis.[29—31] The Euphorbiaceae and Thymelaeaceae are known to include some tiglians and daphnanes, and one tiglian, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), is known to have an effect on hair growth.[32,33] Because daphne factor F3, which we found to be the active ingredient of *H. crepitans*, is classified as a daphnane, it was expected to have some biological effects similar to TPA. Unlike *H. crepitans* from Peru, however, *W. retusa* which includes not only daphne factor F3 but also some daphnanes[25,34,35] did not affect hair growth. *H. crepitans* from Brazil, too, didn't affect hair growth. The daphne factor F3 contents of *W. retusa* and *H. crepitans* from Brazil were very low compared to the daphne factor F3 content of *H. crepitans* from Peru (about one thirtieth and below the detection limit, respectively). These results suggest that daphne factor F3 has a specific effect on hair growth that is different from the effects of other daphnanes.

To clarify the mechanism of the hair growth effect of *H. crepitans*, further investigations are necessary. In particular, to evaluate the NT-4 expression in hair follicles of DHT-implanted mice treated with *H. crepitans* is a matter of high priority. Moreover, to demonstrate that *H. crepitans* inhibits not AR activity but NT-4 activity, supplemental experiments are essential. If *H. crepitans* directly inhibits the transcription of AR, the NT-4 inhibitory activity is one of mechanisms of *H. crepitans* because androgen and AR complex is well known to control the transcriptions of many growth factors. It would therefore be interesting to see whether *H. crepitans* affects the expressions of growth factors controlled by AR, for example IGF-1,[7] TGF-β[8,9] and DKK-1.[10]

On the other hand, BDNF, a neurotrophin and an inducer of catagen like NT-4,[11,13,16] was reported to up-regulate TGF-β2 gene transcription in human anagen hair follicles.[36] If the transcription of BDNF is also controlled by AR, BDNF might play a key role in the up-regulation of TGF-β2 transcription by AR. Considering the effect of TGF-β2 on the hair cycle, we think that further investigations of the crosstalk between neurotrophin signaling and TGF-β signaling would be very interesting.

When the compounds having NT-4 inhibitory activity, such as *H. crepitans* extract and daphne factor F3, are used as therapy for AGA, their effects for other tissues need to be considered. The reason for this is that neurotrophins mediate the survival, differentiation and growth by binding to the tyrosine kinase receptor Trks, and they are the key factors maintaining the function in the Trks expressing cells, such as nerve cells,[12] retinal cells,[37] and so on. In addition, diterpene esters of the daphnane and tiglian type are known to have skin irritation and tumor promoting effect.[24] Therefore, safety evaluation of these compounds is required.

In this study, we found that the transcriptional activity of NT-4 which accelerates the induction of catagen was controlled by AR. We also found that *H. crepitans* from Peru has NT-4 inhibitory activity and a significant effect on hair regrowth in DHT-implanted mice and that daphne factor F3 is the active ingredient included in *H. crepitans*. These findings will, we hope, be contributory to therapy for AGA.

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