"Recombinant Filaggrin 2 Ameliorates DHT-Induced Damage in Dermal Papilla Cells (DPCs)"

Chanhua Zhou [1], Yadong Huang [1,*], Xiaopeng Huang [1], Ziyi Huang [1]

[1] R&D Department, Tyran（Guangzhou）Biotechnology Co., Ltd., Guangzhou, China

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1 Introduction

Androgenetic alopecia (AGA), also termed seborrheic alopecia (SA), is a common cosmetically distressing dermatological disorder. In traditional Chinese medicine, it corresponds to conditions such as Bai Xie Feng (scalp desquamation), Zhu Fa Xuan (hair moth tinea), and Fa Zhu Tuo Fa (moth-eaten alopecia), as documented in Waitai Miyao (Tang Dynasty), which described symptoms of "white dandruff" and "pruritus" akin to modern AGA characteristics[1,2]. AGA is an androgen-dependent, polygenic hereditary disorder characterized by follicular miniaturization, excessive scalp sebum production, pruritus, and progressive hair thinning[3–6]. A hallmark of AGA is elevated local dihydrotestosterone (DHT) levels, which dysregulate hair cycling via activation of downstream signaling pathways mediated by 5α-reductase[7,8].

While minoxidil remains a frontline therapy for AGA by targeting DHT, its utility is limited by adverse effects including scalp irritation, xerosis, erythema, and hypertrichosis[9,10]. Moreover, emerging evidence implicates chronic scalp inflammation—driven by seborrheic dermatitis, microbial dysbiosis (e.g., Malassezia overgrowth), and trauma from scratching— in disrupting follicular stem cell niches and accelerating catagen entry[11–16]. Crucially, epidermal barrier integrity plays a central role in maintaining scalp homeostasis. Barrier dysfunction increases transepidermal water loss, facilitates pathogen penetration, and amplifies inflammatory cascades, ultimately perturbing hair follicle cycling[17–19].

Dermal papilla cells (DPCs), the key regulators of follicular niche homeostasis, are critically impaired in AGA. Functional DPCs are indispensable for normal anagen initiation and cycle progression[20–24]. Notably, filaggrin (FLG), a structural protein essential for natural moisturizing factor (NMF) synthesis, epidermal barrier function, and antimicrobial defense[25–28], has emerged as a pivotal player. FLG mutations are strongly associated with ichthyosis vulgaris, atopic dermatitis (AD), and alopecia areata[29–32]. Preclinical studies reveal that FLG2 deficiency exacerbates barrier defects, induces parakeratosis, elevates skin pH, and disrupts follicular microenvironmental stability—pathological features mirroring AGA-associated scalp xerosis and pruritus[33–36]. Intriguingly, the C-terminal domain of FLG2 exhibits selective antimicrobial activity against Pseudomonas aeruginosa,

suggesting dual barrier-protective and antimicrobial roles[37].Our prior work demonstrates that recombinant FLG2 (rFLG2) mitigates UV-induced oxidative stress by suppressing ROS generation and downregulating proinflammatory cytokines (IL-10, IL-1β)[40]. Building on this, we investigated rFLG2's effects on DHT-injured DPCs. DHT treatment significantly reduced endogenous FLG expression, impairing DPC proliferation, migration, adhesion, and cell cycle progression while inducing apoptosis. Remarkably, rFLG2 supplementation rescued DHT-induced cytotoxicity and restored FLG2 levels. These findings position rFLG2 as a novel therapeutic candidate for AGA, capable of simultaneously addressing androgen-driven follicular regression and barrier dysfunction.

In summary, androgenetic alopecia represents a multifactorial disorder involving aberrant androgen signaling, chronic inflammation, microbial dysbiosis, and compromised epidermal barrier function. Current therapies like minoxidil inadequately address these intertwined mechanisms while exhibiting dose-limiting side effects. Our study identifies filaggrin-2 (FLG2) as a critical nexus linking scalp barrier integrity to follicular homeostasis. By demonstrating that recombinant FLG2 (rFLG2) rescues DHT-induced dermal papilla cell dysfunction—restoring proliferation, migration, and anti-apoptotic activity while counteracting oxidative stress—we propose a novel therapeutic paradigm. This dual-action strategy not only mitigates androgen-driven follicular regression but also reinforces epidermal defense mechanisms, offering a comprehensive approach to AGA management that transcends conventional monotherapies. Our findings underscore the therapeutic potential of targeting barrier-immune-follicular cross-talk in hair loss disorders.

2 Materials and Methods

2.1Materials

Dihydrotestosterone (DHT), minoxidil, fluorescein isothiocyanate (FITC), and basic fibroblast growth factor (bFGF) were obtained from certified biochemical distributors. Cell culture reagents including DMEM/F-12 medium, fetal bovine serum, and penicillin-streptomycin-ampicillin solution were procured from established life science vendors. Transwell chambers (pore size: 0.4 μm) and enzymatic digestion reagents (trypsin) were sourced from specialized laboratory equipment providers.

2.1.1Preparation of cell culture media supplemented with rFLG and DHT

In the cellular experiment, DPCs were exposed to a medium containing a specific concentration of DHT, resulting in cellular damage. Subsequently, varying concentrations of rFLG2 were introduced into the medium. Based on the preliminary experiment, the concentration of DHT was set at 35 nmol/mL, and the effects of different concentrations of rFLG2 (0, 1, 5, 12.5, and 25 μg/mL) on the viability of DPCs were systematically evaluated.

2.1.2Cell proliferation assay

DPCs were seeded in 96-well plates at a density of 5×10^3 cells/well. Cells were initially cultured in complete medium for 4 h and then starved overnight using medium containing 1% FBS. Subsequently, all groups except for the control group were exposed to cellular damage induced by the addition of 35 nmol/ml DHT and incubated with different concentrations of rFLG2 solution. After 24 h, CCK-8 was added to each well and incubated

for two hours. Subsequently, the absorbance at a wavelength of 450 nm was measured using a microplate reader.

2.1.3Cell migration assay

DPCs were seeded into 12-well plates at a density of 3×10^5 cells/well, cultured with complete medium containing 10% FBS to reach approximately 80% confluence.
The cells were then scraped using the tip of a 200 μL micropipette perpendicular to the parallel line, and four fields of view were randomly captured with a microscope. Except for the control group, 35 nmol/ml DHT was added to induce cellular damage. Subsequently, various concentrations of rFLG2 were incorporated into the culture medium, and the cells were incubated for 48 hours. Images were captured at 0, 24, and 48 hours using a microscope. Within each group, four random fields of view were selected for analysis.The cell migration rate was quantified by Image-J software.

2.1.4Cell adhesion assay

The 12-well plates were pre-coated with DEME-F/12 medium supplemented with varying concentrations of rFLG2, along with a control group, and incubated at 4 °C. Once the liquid in the 12 wells had dried, DPCs were seeded into the plates at a density of 2×10^4 cells/well. Following a two-hour incubation, non-adherent cells were rinsed away by PBS. The cells were cultured with a medium containing 1 % FBS for one hour. Except for the control group, 35 nmol/ml DHT was added to induce cellular damage. Subsequently, the cells were stained with crystal violet. For each well, five random fields of view were captured using an optical microscope. Cell numbers in each group were then quantified using the Image-J software.

2.1.5Cell apoptosis assay and cell cycle assay were performed

DPCs were seeded into 6-well plates at a density of 4×10^5 cells/well, cultured with complete medium containing 10% FBS to reach approximately 70% confluence. Additionally, serum-free starvation was then performed for more than 24 h to reset the cell cycle.Except for the control group, 35 nmol/ml DHT was added to induce cellular damage. Subsequently, various concentrations of rFLG2 were incorporated into the culture medium, and the cells were incubated for 24 hours.Flow cytometry was used after staining with Annexin V-FITC/PI Apoptosis Detection kit and Cell cycle and Apoptosis Detection kit. The live and dead staining status of cells was detected by Calcein/PI cell viability and cytotoxicity assay kit, and five fields were randomly captured with a microscope.

2.1.6Quantification of Intracellular ATP Levels

After the cells were treated as described in Section 2.1.1 , the intracellular adenosine 5'-triphosphate (ATP) level was quantified using an ATP detection kit. Chemiluminescent signals were measured withHybrid Multi-Mode Microplate Reader.

2.1.7Sequencing

After the cells were treated as described in Section 2.1.1 , DPCs were washed twice with PBS and total RNA was extracted from the cells using Trizol reagent. mRNA sequencing was completed by Technology.

2.1.8Western Blot Assay

Treated cells were washed with PBS and subsequently lysed using RIPA buffer. The resulting lysates were centrifuged at 12,000× g rpm for 30 minutes, and the supernatants were carefully collected and quantified using the BCA Protein Assay Kit. The isolated proteins were combined with the SDS loading buffer and subsequently heated at 100°C for 10 minutes. The protein samples were then resolved using SDS-PAGE and electrotransferred onto PVDF membranes. The membrane was blocked with 5% skim milk at room temperature for 1 h, then incubated with the primary antibodies against ERK1/2, p-ERK1/2, Bax, Bcl-2, caspase-1, p38, and P-p38 at 4 °C overnight. Subsequently, it was incubated with HRP-conjugated secondary antibodies at room temperature for 1 h. Finally, the immunoreactive bands were detected using the enhanced chemiluminescence (ECL) reagent.

2.1.9Quantitative real-time PCR (qRT-PCR)

The mRNA expression levels of the genes were analyzed using a CFX96 real-time PCR system. The primer sequences used for the analysis were as follows:

Gene Name	Sequence
Mus-Ki67-F	TGCCTCAGATGGCTCAAAGA
Mus-Ki67-R	TCTGCCAGTGTGCTGTTCTA
Mus-TGF-β-F	TTGCTTCAGCTCCACAGAGA
Mus-TGF-β-R	CAGAAGTTGGCATGGTAGCC
Mus-VEGF-F	TTGAGACCCTGGTGGACATC
Mus-VEGF-R	GGGCTTCATCGTTACAGCAG
Mus -Gapdh-F	GGAGAGTGTTTCCTCGTCCC
Mus -Gapdh-R	ATGAAGGGGTCGTTGATGGC

2.1.10Statistical Analysis

Data analysis was conducted using GraphPad Prism 9.5.1 software. All data were expressed as means ± standard deviation. Student's t-tests were used for comparisons between two samples, while comparisons among multiple groups were performed using one-way analysis of variance (ANOVA).P-values less than 0.05 were considered statistically significant and labeled as follows: *$p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3 Results

3.1 rFLG2 Restores DPC Migration and Adhesion Capacity Under DHT Stress

3.1.1rFLG2 ameliorates DHT-induced DPCs damage in vitro.

In the present study, the bioactivity of rFLG2 was investigated in vitro, with a focus on its potential to mitigate DHT-induced damage in DPCs and enhance their proliferation, adhesion, and migration capabilities. Wound healing assay showed that rFLG2 could significantly

improve cell migration induced by DHT (Fig. 7A). After 24 hours, the cell migration rate in DHT-induced group exhibited significant differences compared to those of the 25, 12.5, and 5 μg/ml rFLG2 treatment groups. However, no significant difference was observed between the treated groups and the control group (Fig. 7B). After 48 hours, the migration rate of DHT-induced cells differed significantly between the 25μg/ml and 12.5μg/ml rFLG2 groups. No significant difference was observed between the control group and the rFLG2 groups, indicating that rFLG2 may improve DHT-induced migration impairment (Fig. 7C). Accelerated wound closure was further substantiated by the enhanced cell migration observed in DPCs. The adhesion assay demonstrated that rFLG2 significantly enhanced the adhesion capacity of DPCs (Fig. 7D) (Fig. 7E). The concentrations of rFLG2 at 25, 12.5, 6.25, 3.125, and 1.56 μg/ml were found to effectively mitigate DHT-induced injury. Notably, the concentrations of rFLG2 at 25 μg/ml and 12.5 μg/ml exhibited significant differences compared to the model group, demonstrating a pronounced promotion of cell proliferation (Fig. 7F). Compared with the control group, the DHT injury group could significantly promote DPC cells into early apoptosis, and the number of apoptosis cells in the DHT injury group was significantly increased (Fig. 7G). Compared with the DHT injury group, the rFLG2 treatment groups (25, 12.5, and 6.25 μg/ml) significantly reduced DPC cell apoptosis. Following rFLG2 treatment, the level of apoptosis was restored to a degree that exhibited no significant difference compared with the control group (Fig. 7H). The high expression of Ki67 indicates that the hair follicle stem cells are in the growth phase for a long time, while the high expression of TGF-β indicates that the hair follicle stem cells enter the regression phase, at which time the hair follicle begins to atrophy and undergo necrosis[59]. The results of real-time fluorescent quantitative polymerase chain reaction (PCR) demonstrated that the expression level of Ki67 in the cells was significantly upregulated following rFLG treatment, exhibiting a dose-dependent increasing trend with higher concentrations of rFLG (Fig. 7I). Conversely, the expression of TGF-β was markedly downregulated .
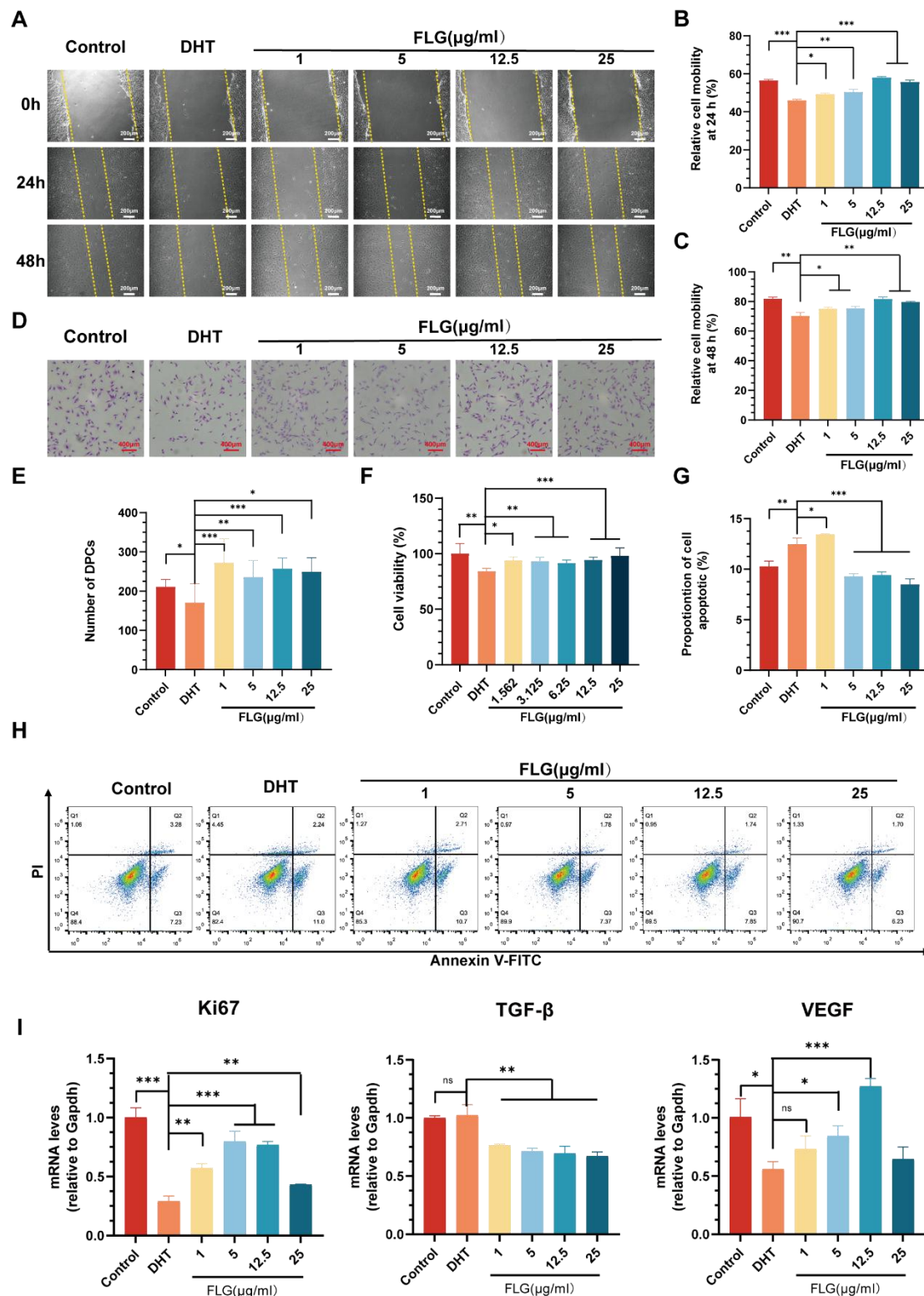
Figure 1. rFLG2 ameliorates DHT-induced DPCs damage in vitro.(A, B, C) Representative images of DPCs scratch assay (100×) and quantitative analysis of migration rate per field of view. Scale bar, 200 μm. (D, E) Representative images of DPCs adhesion assay (100×) and quantitative analysis of the number of cells per field of view.Scale bar, 400 μm. (F) Proliferation assay of DPCs. (G, H) Determining early apoptosis by flow cytometry Annexin V/PI assay. (I) Quantifying cytokine expression (Ki67, TGF-β， VEGF) in cell. Relative mRNA levels were normalized against the levels of Gapdh. Data were presented as mean ± SD (*p < 0.05, **p < 0.01, and

***p < 0.001).

3.1.2Transcriptomic and Mechanistic Insights into rFLG2 Activity

Moreover，mRNA sequencing analysis was conducted on DPCs that were damaged by DHT, both prior to and following rFLG2 treatment. After treatment with rFLG2, it was observed that 367 genes were up-regulated and 287 genes were down-regulated (Fig. 2A, 2B). Among these genes, we identified those associated with the regulation of hair follicle growth stages, including the MAPK signaling pathway, TGF-β signaling pathway, focal adhesion, and phosphatidylinositol signaling pathway (Fig. 2C).
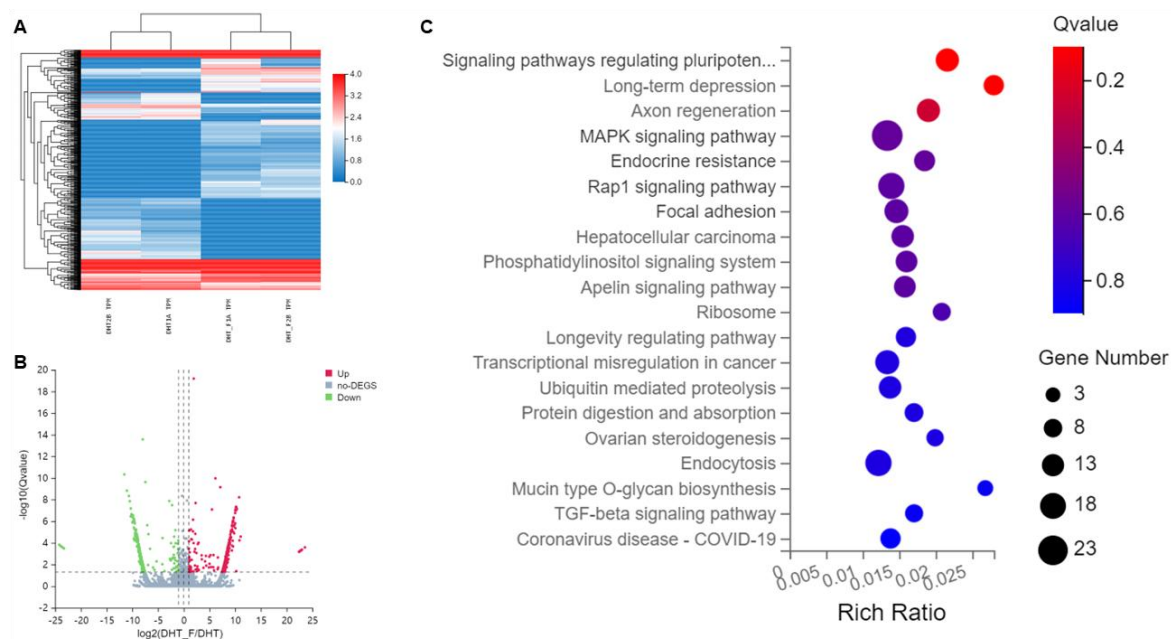

Figure 2. Transcriptomic profiling of DHT-damaged DPCs post rFLG2 treatment. (A) Heatmap depicting differential gene expression between untreated (blue) and rFLG2-treated (red) conditions. (B) Scatter plot illustrating log2 fold changes in gene expression (367 up-regulated and 287 down-regulated genes). (C) Enrichment analysis of signaling pathways linked to hair follicle growth regulation, including MAPK, TGF-β, focal adhesion, and phosphatidylinositol signaling pathways, based on significance (q-value).

To further elucidate the regulatory effect of rFLG2 on DPCs damaged by DHT, we systematically evaluated the differences in the expression levels of proteins related to the MAPK signaling pathway using Western Blot analysis. As shown in Figure, DHT-induced injury to DPCs was characterized by the significant downregulation of p-ERK1/2 and Bcl-2, as well as the upregulation of Bax, caspase-1, and P38[60]. Treatment with rFLG2 can antagonize DHT-induced apoptosis in DPCs by upregulating the expression of p-ERK1/2 and Bcl-2 proteins, while simultaneously inhibiting the expression of Bax and caspase-1 via the MAPK signaling pathway (Fig. 7J). These results support our hypothesis that rFLG2 may play a systemic regulatory role and potentially serve as a treatment for AGA via multiple pathways, thereby improving therapeutic efficacy.
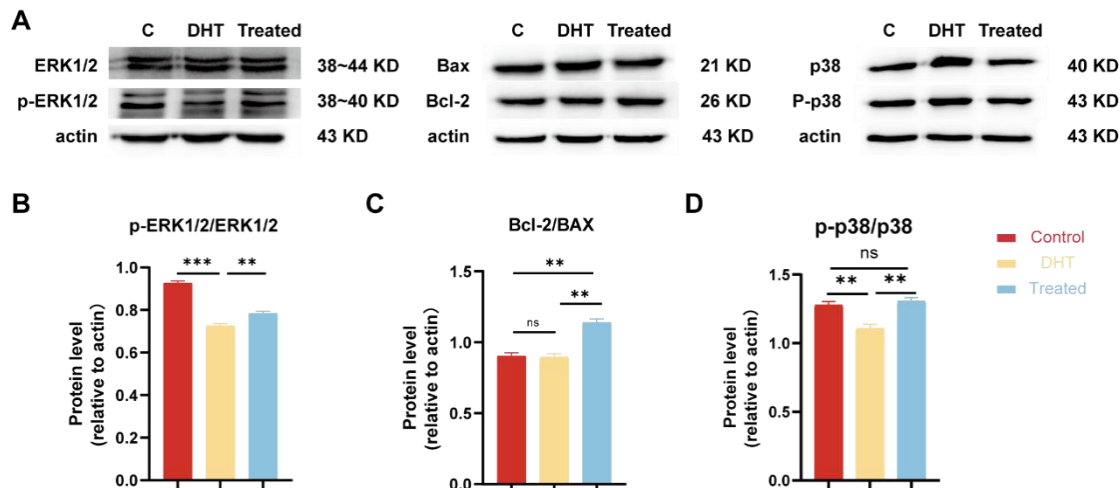
Figure 3. Protein level of ERK1/2, p-ERK1/2, Bax, Bcl-2, p-38 and p38 in DPCs of DHT-induced was analyzed by Western blot (n = 3). Actin antibody was used as control. Data were presented as mean $\pm$ SD (*p < 0.05, **p < 0.01, and ***p < 0.001).

4 Discussion

The escalating prevalence of hair loss, driven by societal stress and lifestyle changes, has intensified public awareness of androgenetic alopecia (AGA)—a condition imposing profound psychological burdens and impairing patients' quality of life [41]. While minoxidil remains a frontline therapy for AGA by inhibiting dihydrotestosterone (DHT), its clinical utility is limited by adverse effects such as scalp irritation, allergic dermatitis, and hypertrichosis, which may paradoxically exacerbate hair loss [42]. These challenges underscore the imperative to develop novel therapeutic strategies that combine hair regrowth promotion with enhanced biocompatibility and barrier repair.

AGA pathogenesis is characterized by follicular miniaturization, driven by genetic predisposition and perifollicular microinflammation. Immune cell infiltration and cytokine release disrupt the hair follicle stem cell niche, promoting fibrosis and irreversible miniaturization [43,44]. Androgen receptors further exacerbate this process by suppressing Wnt/β-catenin signaling and generating negative feedback in Notch pathways [45,46]. Notably, wound healing mechanisms have revealed opportunities for regeneration, with studies demonstrating laser-induced microinjuries can stimulate hair neogenesis during tissue repair [47-48].

Dermal papilla cells (DPCs), central to follicular niche homeostasis, exhibit heightened androgen sensitivity in AGA. DHT-mediated inhibition of Wnt/β-catenin signaling alters DPC secretory profiles, disrupting hair cycle progression [49]. Building on filaggrin's known roles in barrier protection and antimicrobial activity [50,51], our study demonstrates recombinant filaggrin-2 (rFLG2) effectively counteracts DHT-induced DPC dysfunction. rFLG2 restored cellular morphology, enhanced proliferation/migration, and suppressed apoptosis, correlating with increased VEGF/Ki67 expression and reduced TGF-β levels—a cytokine family implicated in catagen progression [52-53]. Mechanistically, rFLG2 augmented intracellular ATP levels, potentially energizing growth factor secretion [54].

While our findings highlight rFLG2's capacity to modulate proliferative markers and follicular activity, limitations exist. Murine models may incompletely recapitulate human follicular dynamics due to interspecies differences in hair cycle synchronization and stem cell regulation [55-58]. Future studies employing ex vivo human skin models will better validate therapeutic potential. Additionally, the interplay between inflammatory pathways and barrier integrity warrants deeper exploration to address AGA's multifactorial nature [59, 60]. Optimization of delivery parameters, including microneedle treatment frequency, remains crucial for balancing clinical efficacy with patient compliance.

5 Conclusion

This study establishes recombinant filaggrin-2 (rFLG2) as a multifunctional therapeutic agent against dihydrotestosterone (DHT)-induced damage in dermal papilla cells (DPCs), a key cellular mechanism underlying androgenetic alopecia (AGA). rFLG2 demonstrated dose-dependent efficacy in rescuing DHT-impaired cellular functions, including migration, adhesion, proliferation, and apoptosis resistance. Mechanistically, rFLG2 modulated follicular cycle-related pathways by upregulating MAPK signaling (via p-ERK1/2 and Bcl-2) and suppressing TGF-β-mediated catagen transition, while concurrently enhancing proliferative activity (Ki67) and mitigating caspase-dependent apoptosis. Transcriptomic profiling further corroborated its systemic regulatory role in focal adhesion and phospholipid signaling. Importantly, rFLG2 restored cellular homeostasis at low concentrations, highlighting its translational potential. When integrated with a hyaluronic acid-based delivery system, rFLG2 offers a synergistic strategy to counteract AGA's multifactorial pathogenesis through cytoprotection, cell cycle regulation, and tissue remodeling. These findings position rFLG2 as a promising candidate for combinatory therapies targeting androgen sensitivity and follicular regeneration, bridging molecular mechanisms with clinical applications for hair loss management.

6 References

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