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ORIGINAL RESEARCH

Human Fibroblast Growth Factor 9 Induces Hair Follicle Cycle Transition via TGF-β/BMP/Smad Pathway

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Background: Fibroblast growth factor 9 (FGF9) is a crucial regulator of hair follicle development; however, the precise regulatory role of FGF9 on the biological behavior of hair follicles remains unclear.

Purpose: Explore the effects of recombinant human fibroblast growth factor (rhFGF9) on hair follicle growth and cycle transition in mice, as well as the associated mechanisms.

Methods: We utilized soluble recombinant human fibroblast growth factor 9 (rhFGF9), expressed in Escherichia coli, and injected it into the dorsal skin of mice. RNA sequencing (RNA-seq) was employed to identify differentially expressed genes and associated key metabolic pathways in skin tissue. Additionally, immunohistochemistry, quantitative PCR (qPCR), and Western blotting techniques were applied to assess the expression of factors related to metabolic pathways across different groups.

Results: RhFGF9 stimulated hair growth by inducing the anagen phase in C57BL/6 N mice. Histological analysis revealed that intradermal injections of rhFGF9 enhanced hair follicle development. Transcriptomic analysis revealed that the mechanism underlying this effect might involve the transforming growth factor-β (TGF-β) signaling pathway. Immunohistochemistry, quantitative PCR, and Western blot analyses further demonstrated the upregulation of TGF-β2, TGF-β3, bone morphogenetic protein 4 (BMP4), and pSmad2 expression in the experimental group.

Conclusion: These results indicate that rhFGF9 might promote hair growth via the TGF-β/BMP/Smad signaling pathway and highlight its potential as a therapeutic option for treating hair loss.

Keywords: fibroblast growth factor 9, hair growth, hair follicle, TGF-β/BMP/Smad pathway

Introduction

Hair loss is an increasingly common aesthetic concern with a continuously increasing prevalence, carrying significant psychosocial consequences. Hair follicles, essential for hair production, undergo a periodic cycle with three distinct phases: anagen, catagen, and telogen.[1] Disruption of this hair follicle cycle results in irregular hair growth and alopecia.[2] Currently, drug options for treating hair loss are limited. Therefore, it is imperative to explore potent medications and mechanisms to promote hair growth, extend the anagen phase of the hair follicle, and ultimately address hair loss.

Fibroblast growth factor (FGF) is essential for numerous biological processes and plays a pivotal role in regulating development, maintaining biological homeostasis, and influencing disease progression.[3] Recently, significant attention has focused on the involvement of the FGF9 subfamily in regulating hair growth and development.[4] However, the precise regulatory effects of FGF9 on hair follicles and its underlying mechanisms remain to be fully elucidated.

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Hair follicle development and cycling depend on a complex network of signaling pathways, with the TGF-β/BMP/Smad pathway playing a key role in intercellular communication. Transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP), both part of the TGF-β superfamily, bind to cell surface receptors. This binding activates intracellular proteins called Smads, which then regulate gene transcription. Recent studies suggest that FGF9 may interact with the TGF-β/BMP/Smad pathway, providing insights into the regulation of hair follicles.[5,6]

This study aimed to investigate the effects of recombinant human FGF9 (rhFGF9) on hair follicle growth and cycle transformation in mice after intradermal injection. Additionally, this study also examined the role of the TGF-β/BMP/Smad signaling pathway in this process, aiming to offer new insights into the potential treatment strategies for hair disorders.

Methods

Animals and Grouping

All the in vivo experiments were conducted on healthy female C57BL/6N mice (8 weeks old) obtained from Beijing Vital River Laboratory Animal Technology, Co., Ltd. The experiments complied with the Guidelines for the Care and Use of Laboratory Animals established by National Institute of Health (NIH). An electric clipper and hair removal cream (Veet, France) were used to remove hair from the dorsal flanks of mice (n = 30), which were then randomly assigned to two groups: a control group and an rhFGF9-treated group (n = 15 per group). The high-purity rhFGF9 utilized in our study was sourced from an engineered strain that effectively expresses soluble rhFGF9 in Escherichia coli. The control group received treatment with phosphate-buffered saline (PBS). After hair removal, the dorsal skin appeared pink, and the hair follicles were in the telogen phase. A single intradermal injection of rhFGF9 (10 μg/mL in 250 μL PBS, 2.5 μg per mouse) or vehicle (250 μL PBS) was administered at multiple points on the dorsal skin of each mouse. Photographs documenting hair growth were taken on days 0, 6, 13, and 21. On days 6, 13, and 21, skin samples were collected by sacrificing five mice per group.

Histological Analyses

After administering either rhFGF9 or control treatments, mouse skin samples were collected, fixed with tissue cell fixative, and embedded in paraffin. Subsequently, the samples were sliced into 5-μm-thick sections and stained with hematoxylin and eosin (HE) following the standard protocols. The measurement method for hair follicles is consistent with the approach adopted in our previous study,[7] and the measurement technique for dermal thickness aligns with the research conducted by Ryu et al.[8]

RNA Sequencing (RNA Seq)

Total RNA was isolated from mouse skin on day 6 post-treatment using the TRIzol kit (Qiagen, Germany), and RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent, USA). After RNA extraction, mRNA was purified with Oligo (dT) beads, fragmented into shorter sequences, and reverse-transcribed into complementary DNA (cDNA). The prepared cDNA library underwent sequencing on the Illumina NovaSeq 6000 platform provided by Gene Denovo Biotechnology Co (Guangzhou, China). Differentially expressed genes (DEGs) were determined, and their enrichment in the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway databases was analyzed. A comprehensive analysis was conducted to identify biochemical metabolic and signal transduction pathways associated with hair growth. The DEGs between the rhFGF9-treated and control groups were identified based on a fold change >1.5 and a FDR <0.05. The RNA-sequencing data has been submitted to the NCBI database, cataloged under BioProject ID PRJNA 1191149.

Quantitative PCR

Total RNA was isolated from mouse skin samples at days 6, 13, and 21 post-treatment using the miRNeasy Mini Kit. The RNA was then reverse-transcribed into cDNA using the Evo M-MLVRT Master Mix kit (Accurate Biology, China). The primers targeting TGF-β2 (F: 5′TCGACATGGATCAGTTTATGCG, R: 5′CCCTGGTACTGTTGTAGATGGA), TGF-β3 (F: 5′

GGCCCTGGACACCAATTACT, R: 5′AGGTTCGTGGACCCATTTCC), BMP4 (F: 5′GAGGAGGAGGAGGAAGAGCA, R: 5′TGCTGCTGAGGTTGAAGAGG), and GAPDH (F: 5′AAATGAGAGAGGCCCAGCTAC, R: 5′CACTGCACAAGAAGATGCG) were used in this study. Quantitative PCR (qPCR) was performed on 384-well plates using the 7900HT Fast qPCR system. Each reaction contained 5 μL of 2× SYBR Green Pro Taq HS Premix, 0.2 μL of ROX Reference Dye, 0.4 μL of primer mix, 500 ng of cDNA, and nuclease-free water, making the total reaction volume of 10 μL. All assays were run in triplicate and repeated three times. Data analysis was conducted using the $2^{-\Delta\Delta Ct}$ method.

Immunohistochemical Staining

Paraffin-embedded sections were deparaffinized and rehydrated through a series of xylene and ethanol washes. Antigen retrieval was performed by autoclaving in sodium citrate buffer or EDTA for 2 minutes. The sections were then blocked with an endogenous peroxidase blocker and a nonspecific staining blocker for 10 minutes at room temperature. The sections were then incubated overnight at 4°C with primary antibodies, including rabbit anti-TGF-β2 (1:180 dilution, ThermoFisher, PA5-86215), anti-BMP4 (1:300 dilution, Abcam, ab155033), anti-Noggin (1:175 dilution, Abcam, ab16054), anti-p-Smad2 (1:2000 dilution, Abcam, ab280888), and anti-Smad4 (1:100 dilution, Abcam, ab40759). The next day, the samples were incubated with respective biotin-labeled secondary antibodies and streptavidin-horseradish peroxidase (HRP). Finally, the tissue sections were stained with DAB and counterstained with hematoxylin. The sections were observed under a microscope and photographed.

Western Blot Analysis

Western blot analysis was conducted to detect proteins associated with hair growth. At each specified time point post-treatment, the mouse skin tissues were lysed in RIPA buffer containing a protein phosphatase inhibitor. The protein concentrations were measured using the BCA method, and 20 μg of protein from each sample was subjected to SDS-PAGE and then transferred onto PVDF membranes. The membranes were incubated with primary antibodies, including rabbit anti-TGF-β2 (1:1000 dilution, Solarbio, K003684P), anti-TGF-β3 (1:1000 dilution, Solarbio, K005854P), anti-p-Smad2 (1:1000 dilution, Abcam, ab280888), anti-Smad2 (1:1000 dilution, Abcam, ab33875), anti-Smad4 (1:5000 dilution, Abcam, ab40759), and anti-Tubulin (1:500 dilution, Abcam, ab6046), followed by incubation with HRP-labeled goat anti-rabbit IgG (1:10000 dilution, Abcam, ab6721) as the secondary antibody. Signal detection was performed using the Tanon imaging system, and grayscale analysis of the protein bands was conducted using ImageJ software.

Statistical Analysis

The experimental data were analyzed using GraphPad Prism software. Results are presented as means ± SD, calculated from three or more independent experimental replicates. Statistical comparisons were conducted using one-way or two-way analysis of variance (ANOVA), followed by Dunnett's post hoc test. A P-value of <0.05 was considered statistically significant.

Results

Impact of rhFGF9 on Hair Growth

Black pigmentation serves as a marker for hair follicles transitioning from the telogen to the anagen phase.[9] At specified intervals, hair growth was assessed by observing changes in skin color. On day 6, the dorsal skin of mice treated with rhFGF9 showed visible black pigmentation, with prominent dark spots, in contrast to minimal darkening in the control group. By day 13, the majority of rhFGF9-treated mice had entered the anagen phase, exhibiting significant hair growth, whereas the control group displayed only a slight increase in pigmentation. By day 21, nearly complete hair growth was observed in the rhFGF9-treated mice, with most of the dorsal skin fully darkened. In contrast, the control group exhibited significantly less darkened skin (Figure 1).

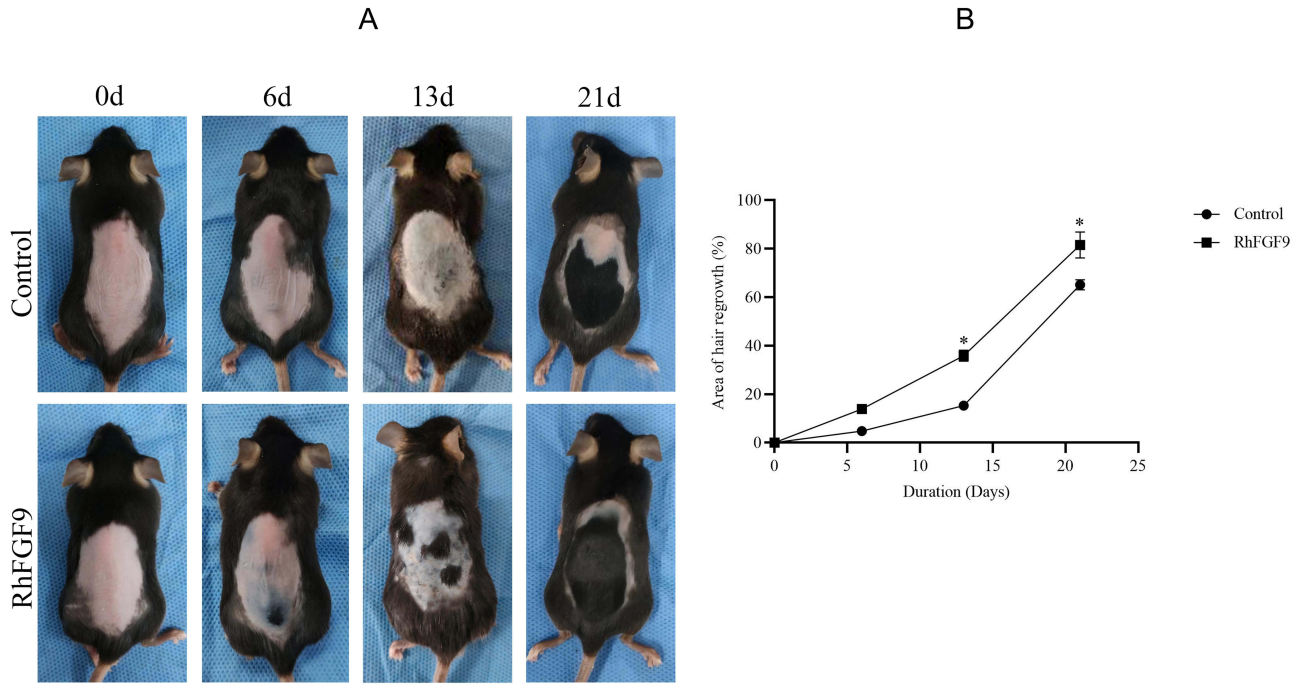

Figure 1 Impact of rhFGF9 on hair growth in C57BL/6N mice. (**A**) Representative images were obtained on days 0, 6, 13, and 21 after the application of either rhFGF9 or PBS to the shaved dorsal skin. (**B**) Quantification of hair regrowth area is expressed as a percentage. * $P < 0.05$.

Histological Observation of Hair Follicles

Hair follicles vary in size and shape depending on their developmental stage, with mature follicles being larger and positioned closer to the subcutis.[10] By day 6 post-treatment, hair follicles in the control group remained mostly in the telogen phase, situated within the dermis. In contrast, follicles in the rhFGF9-treated group showed increased length and number compared to controls, gradually migrating downward and entering the early anagen phase. On days 13 and 21, hair follicles on the dorsal skin of both groups advanced into the growth phase. However, in the rhFGF9 group, follicles extended deeper into the subcutaneous layer and displayed greater size, maturity, and length as compared to those in the control group (Figure 2A and B). The rhFGF9-treated group also showed a significant increase in skin thickness (Figure 2C).

TGF-β Might Be a Target for rhFGF9 in Hair Growth

The RNA-seq analysis of skin tissues from both the groups was performed with a minimum of three replicates for each group. The results showed a total of 126 DEGs, which included 50 upregulated and 76 downregulated genes (Figure 3A). In order to better understand the significance of these DEGs in rhFGF9-induced hair growth, functional enrichment analysis was performed using GO and KEGG. GO analysis identified three main categories: biological process, molecular function, and cellular component. In addition, KEGG pathway analysis indicated that the DEGs were associated with various processes, including metabolism, human diseases, cellular activities, environmental information processing, genetic information handling, and biological systems. Notably, the TGF-β signaling pathway was among the most significantly enriched metabolic pathways (Figure 3B and C).

RhFGF9 Could Activate TGF-β/BMP/Smad Signaling Pathway

In order to validate the RNA-seq findings, key factors from the TGF-β and BMP subfamilies within the TGF-β superfamily were selected for qPCR analysis. The results demonstrated that mRNA levels of TGF-β2, TGF-β3, and BMP4 were upregulated to varying extents at different time points post-treatment (Figure 4A–C), with significant increases observed on day 13 following rhFGF9 administration.

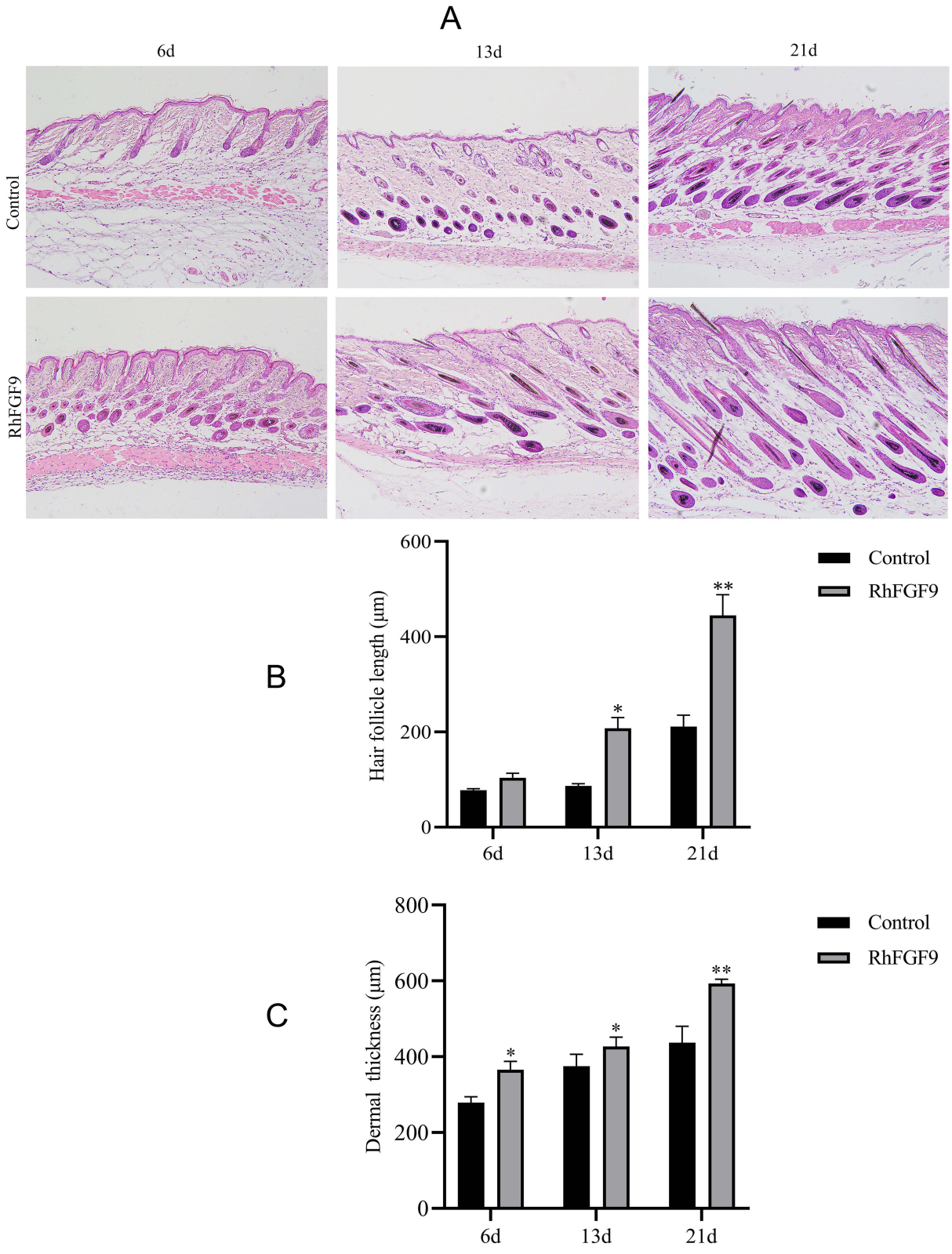

Figure 2 Impact of rhFGF9 on hair follicles in C57BL/6N mouse model. (**A**) Histological analysis of skin sections across experimental groups (scale bar, 200 μm). (**B**) Variation in hair follicle length among the different groups. (**C**) Differences in skin thickness among different groups. Data are presented as means ± SD, **P* <0.05 and ***P* <0.01 compared to the control group.

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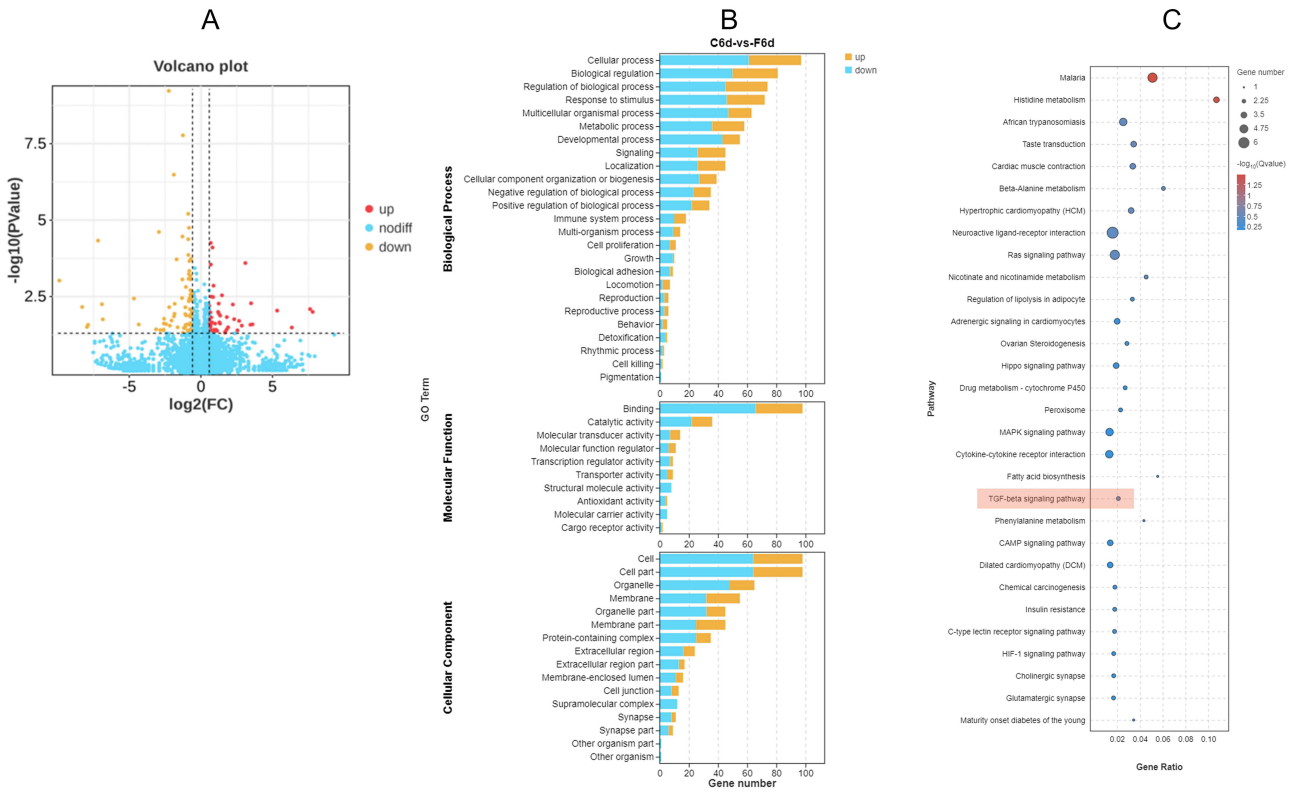

Figure 3 Transcriptomic analysis of differential gene expression in rhFGF9-treated and control groups. (**A**) Volcano plot showing differential gene expression in the dorsal skin of C57BL/6N mice 6 days post-rhFGF9 treatment, with downregulated genes marked in yellow and upregulated genes in red. (**B**) Histogram displaying GO enrichment across biological processes, molecular functions, and cellular composition. (**C**) KEGG enrichment analysis of DEGs highlighting the top 30 significantly enriched pathways, ranked by Q-value, including the TGF-β signaling pathway.

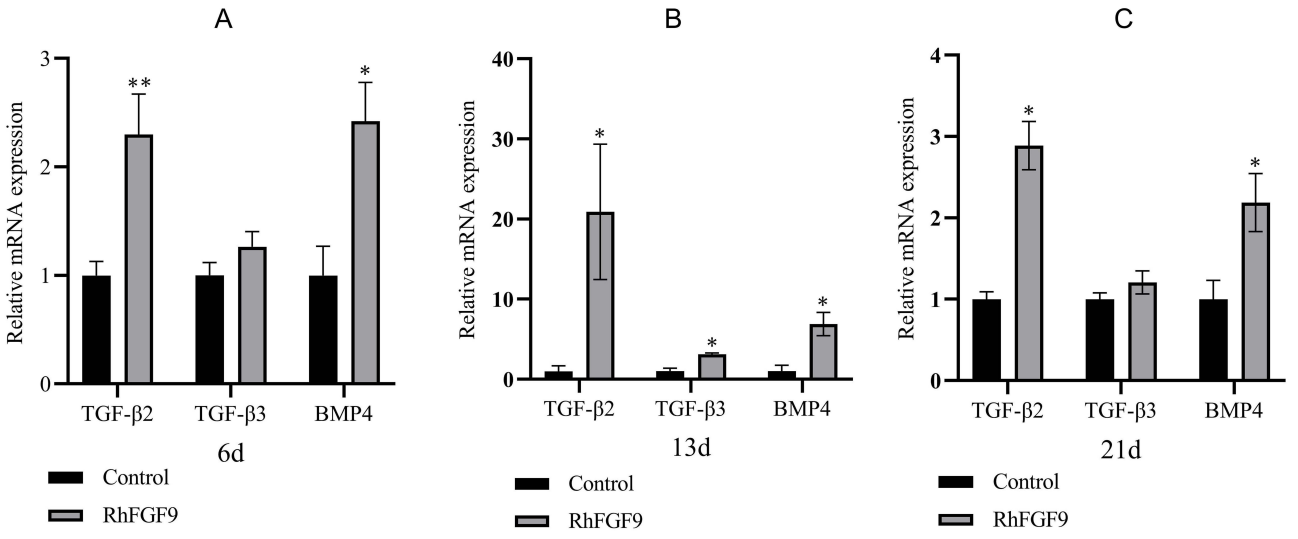

Figure 4 Effect of rhFGF9 on mRNA expression of factors associated with TGF-β/BMP pathway. (**A**) Relative mRNA level of TGF-β2, TGF-β3, and BMP4 in each group on day 6 post-injection. (**B**) Relative mRNA level of TGF-β2, TGF-β3, and BMP4 in each group on day 13 post-injection. (**C**) Relative mRNA level of TGF-β2, TGF-β3, and BMP4 in each group on day 21 post-injection. *P <0.05 and **P <0.01.

Immunohistochemistry and Western blot analyses were conducted to evaluate the expression levels of TGF-β/BMP/Smad signaling proteins in the dorsal skin of both mouse groups. Immunohistochemistry revealed a significant increase in TGF-β2 protein expression by day 13 post-rhFGF9 treatment, with BMP4 protein levels notably higher on days 6 and 13, which aligned with the qPCR results (Figure 5A and B). Additionally, Smad2 phosphorylation, a downstream marker

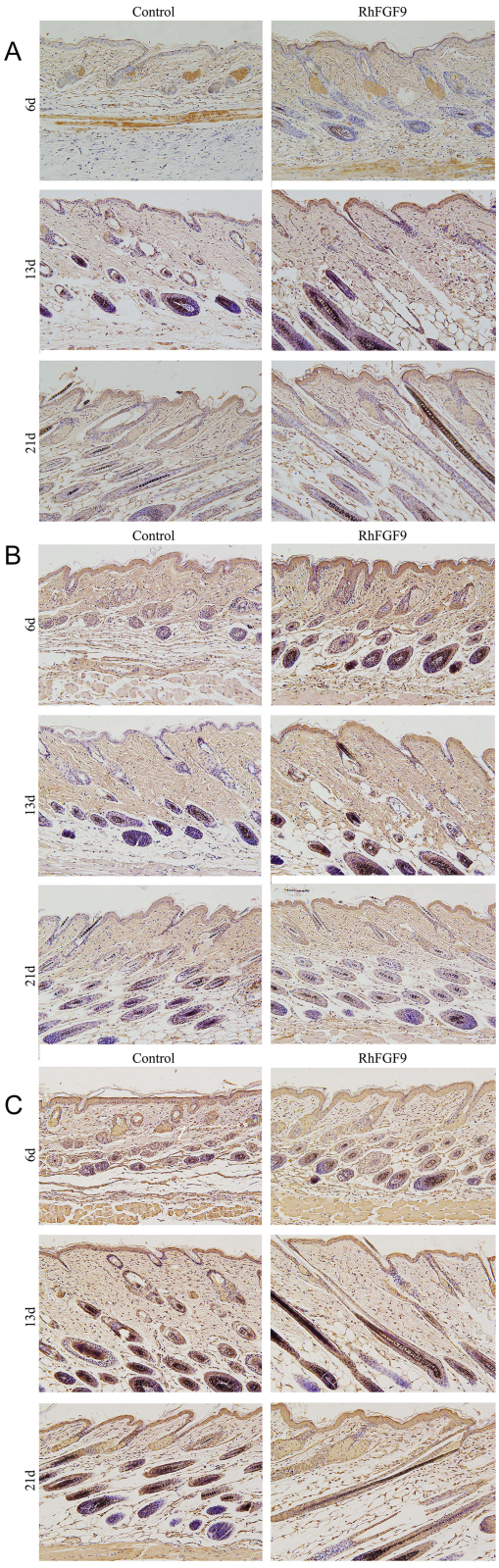

Figure 5 Continued.

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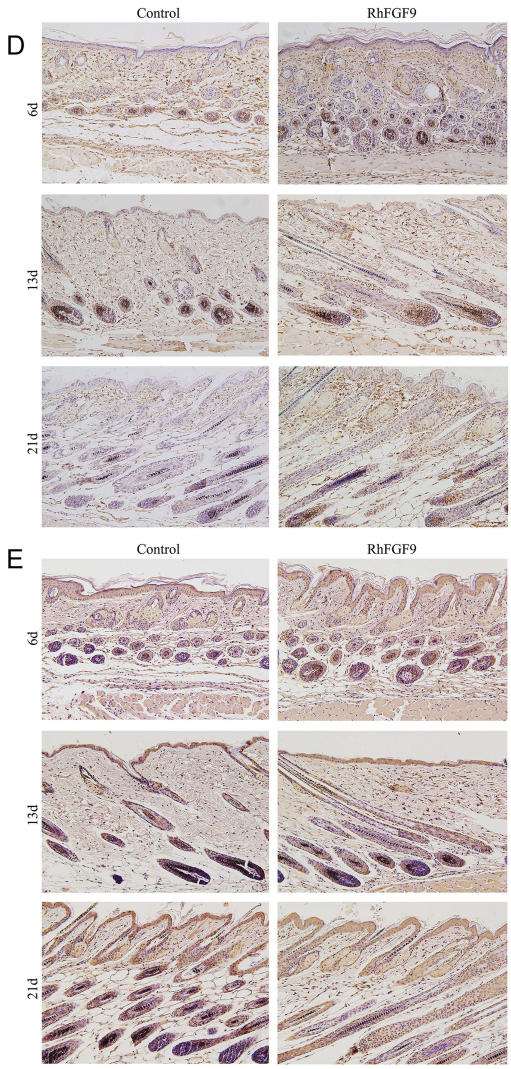

Figure 5 DAB staining of factors in the TGF-β/BMP/Smad pathway following rhFGF9 treatment. (**A**) Expression of TGF-β2 after rhFGF9 injection. (**B**) Expression of BMP4 after rhFGF9 injection. (**C**) Expression of Noggin following rhFGF9 injection. (**D**) Expression of p-Smad2 after rhFGF9 injection. (**E**) Expression of Smad4 after rhFGF9 injection. All digital photomicrographs were captured from representative regions at 200X magnification.

of TGF-β activation, was elevated (Figure 5D), while both Smad4 expression and BMP antagonist Noggin were reduced (Figure 5C and E). Western blot results corroborated these findings, showing marked upregulation of TGF-β2 and TGF-β3 proteins on day 13 post-rhFGF9 treatment, consistent with qPCR observations (Figure 6A–C). The phosphorylation of Smad2 was also elevated at multiple time points (Figure 6A and D), whereas Smad4 expression was found to be downregulated (Figure 6A and E). Collectively, these results suggested that rhFGF9 treatment could activate the TGF-β/BMP/Smad pathway, potentially contributing to enhanced hair growth.

Discussion

Hair loss affects approximately 147 million individuals worldwide, with nearly 50% of men and women experiencing some form of pattern baldness at various stages in life.[11] Despite the availability of treatments, current options remain limited, leading researchers to investigate alternative therapeutic strategies.

FGFs and their receptors are expressed in both the skin and hair follicles, influencing hair follicle development. As a treatment for hair loss, platelet-rich plasma (PRP) is rich in growth factors, and these growth factors play a crucial role in

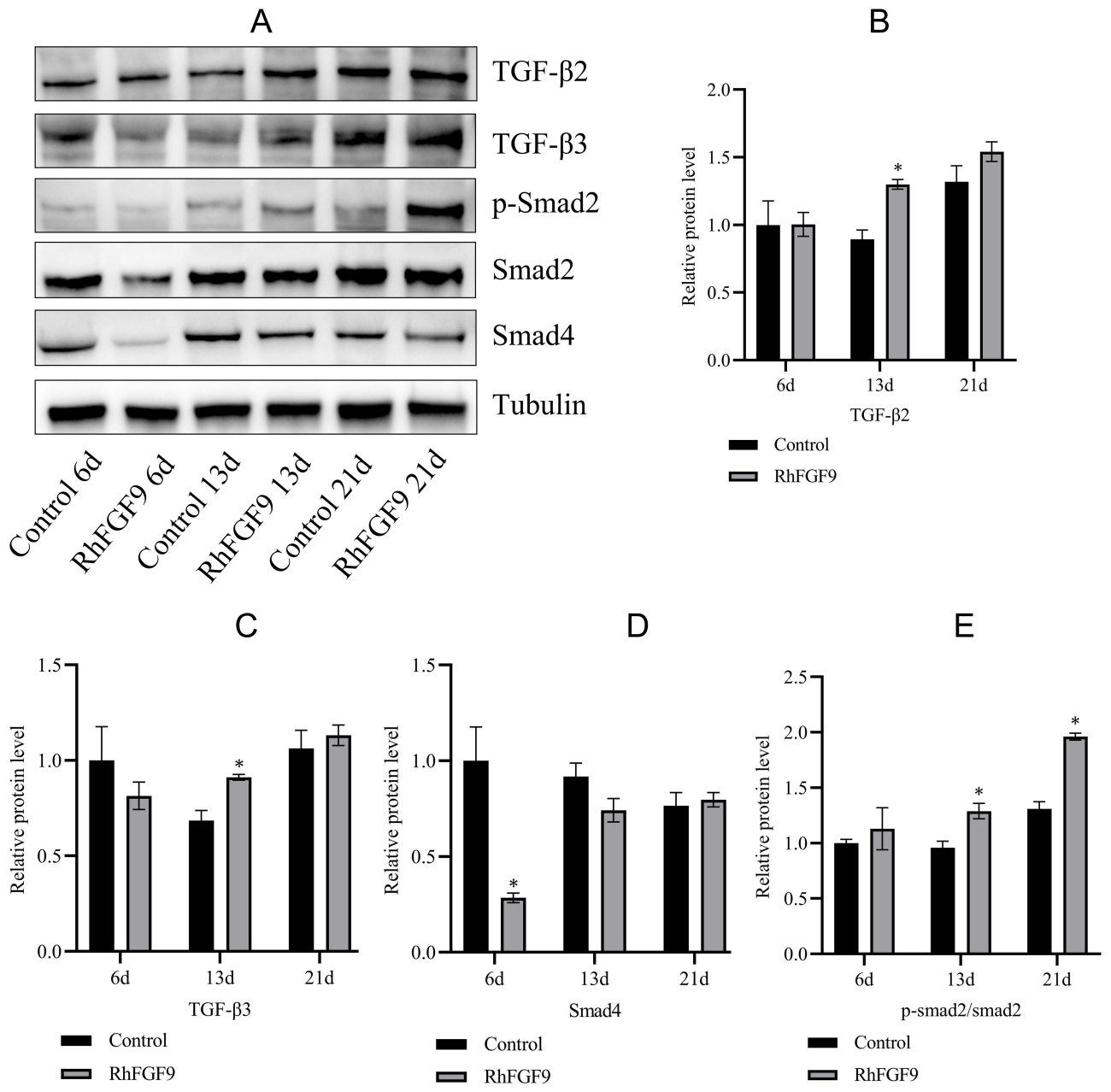

Figure 6 Effects of rhFGF9 on protein levels associated with the TGF-β/BMP signaling pathway. (**A**) Comparative levels of proteins associated with the TGF-β/BMP signaling pathway. (**B**) Expression levels of TGF-β2 protein. (**C**) Expression levels of TGF-β3 protein. (**D**) Expression levels of Smad4 protein. (**E**) Expression levels of p-Smad2/Smad2 protein. *$P < 0.05$.

tissue regeneration, which serves as an important therapeutic mechanism of PRP in hair restoration. Various growth factors, including FGF1, FGF2, and FGF10, have shown efficacy in promoting hair growth by driving hair follicles into the anagen phase.[9] Additionally, Gentile et al found that PRP promotes the differentiation of hair follicle stem cells by upregulating the FGF7 signaling pathway.[12] Kinoshita-Ise et al further discovered that FGF7 is less effective than FGF9 in promoting hair follicle regeneration, moreover, FGF9 has been identified as a supportive agent in post-traumatic hair follicle regeneration and a critical regulator of hair follicle morphogenesis.[13] Studies by Jia indicate that FGF9 promotes dermal papilla cell proliferation and accelerates cell cycle progression.[14] Our previous research showed that FGF20, a member of the FGF9 subfamily, regulates hair follicle growth and regeneration.[15] We have discovered that FGF9 exerts a

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more pronounced positive effect on hair follicle growth compared to FGF20. Indeed, FGF9 holds greater promise for the treatment of hair loss.

We obtained high-purity rhFGF9 by utilizing an engineered strain of Escherichia coli that efficiently expresses soluble rhFGF9. Previous studies have demonstrated that this purified form of rhFGF9 regulates cell proliferation and migration in human liver cancer cell lines.[16] The findings of this study demonstrated that rhFGF9 could promote hair follicle growth and accelerate the transition through hair cycle phases in mice. These results aligned with those of Cai et al[17] who showed that the topical application of oil-body-bound oleosin-rhFGF9 could enhance hair growth. In this study, the dorsal skin depilation model, a commonly used method for investigating hair dynamics, was utilized. Following complete depilation, both the rhFGF9-treated and control groups received uniform intradermal injections of rhFGF9 and PBS, respectively, at multiple sites. The rhFGF9-treated group mice exhibited earlier skin darkening and entered the anagen phase sooner than those in the control group. Additionally, the hair growth area in the rhFGF9-treated group was significantly larger on days 13 and 21 post-injection. HE staining revealed that hair follicles in the rhFGF9-treated group were longer, with larger hair bulbs, increased melanin secretion, and thicker skin compared to the control group, indicating an earlier entry into the growth phase.

In order to investigate the mechanisms by which rhFGF9 regulated hair follicles, transcriptomic analysis was performed on skin samples from two groups of mice. KEGG enrichment analysis of DEGs indicated an involvement of the TGF-β signaling pathway. Among the members of the TGF-β superfamily, TGF-β1 and TGF-β2, along with BMP2, BMP4, and BMP6 from the BMP subfamily, are among the most extensively studied proteins.[18] In the canonical TGF-β/BMP signaling pathway, these proteins exert their effects by binding to specific downstream Smad proteins. The TGF-β/BMP/Smad signaling pathway is involved in a broad range of biological processes in mammals and is closely linked to hair growth and development.[19,20]

TGF-β2, synthesized by dermal papilla cells, is crucial for hair follicle morphogenesis and the activation of hair follicle stem cells (HFSCs). It promotes pSmad2 activation in HFSCs and plays a pivotal role in the transition from telogen to anagen phases. Hair follicle development was delayed in TGF-β2 knockout mice.[21] Studies suggested that certain hair loss treatments could enhance TGF-β2 signaling,[11] whereas TGF-β2 inhibition in human dermal papilla cells could significantly impair hair follicle development and maturation.[22] Compared to TGF-β2, TGF-β3 has been less thoroughly investigated in hair research. Available evidence indicates that TGF-β3, primarily produced by skin T regulatory (Treg) cells, facilitates HFSC activation and proliferation through Smad2/3 signaling.[23] In this study, rhFGF9 was observed to upregulate TGF-β2, TGF-β3, and pSmad2 expression, potentially contributing to the positive effects of rhFGF9 on hair growth.

BMP4 plays multiple roles in hair growth and development regulation. Although often considered a negative regulator of hair growth,[24] its beneficial contributions are well-established. BMP4 demonstrates significant biological functions and potential applications, particularly in maintaining hair homeostasis and promoting regeneration.[25] Research has shown that BMP4 supports the proliferation and migration of dermal papilla cells and melanocytes.[26,27] Teng et al reported that icariin could promote hair follicle formation by upregulating BMP4 signaling.[28] The current findings indicated that rhFGF9 upregulated BMP4 while downregulating the BMP antagonist Noggin, potentially clarifying the mechanism of action of rhFGF9. As a downstream protein, Smad4 influences hair follicle differentiation and growth by mediating the TGF-β/BMP signaling pathway.[29] Following rhFGF9 application, the observed downregulation of Smad4 protein may be linked to TGF-β/BMP pathway activation.

The involvement of rhFGF9 in TGF-β/BMP signaling has been documented in prior studies. FGF9 affects the secretion of associated proteins by inhibiting the TGF-β1/Smad signaling pathway.[5] Notably, FGF9 protein levels increase in fibroblasts when exposed to elevated TGF-β1 levels.[30] Furthermore, FGF9 enhances bone cell differentiation by promoting BMP signaling activity, a critical process in bone development.[31] Exploring the interaction between FGF9 and TGF-β/BMP signaling could significantly advance regenerative medicine research, particularly in the context of hair regeneration.

Our study acknowledges several limitations. First, the newly produced rhFGF9 exhibits a short validity period and is relatively unstable This issue could potentially be addressed by developing advanced drug delivery systems capable of effectively preserving rhFGF9 and extending its shelf life. Secondly, our experiments indicate that rhFGF9 can promote

hair growth at low doses; however, we have observed through numerous experiments that rhFGF9 does not significantly enhance hair growth under frequent stimulation at high concentrations and may even inhibit hair growth. The specific mechanisms underlying these observations warrant further investigation.

Conclusion

In summary, this study demonstrated that rhFGF9 might facilitate the transition of hair follicles from the telogen phase to the anagen phase, thereby promoting hair growth through the activation of the TGF-β/BMP/Smad signaling pathway. Furthermore, as FGF9 is susceptible to degradation by various enzymes, strategies to increase its stability might improve its efficacy in treating hair loss. Overall, rhFGF9 might be a promising candidate for the development of novel therapies targeting hair loss disorders.

Abbreviations

RhFGF9, Recombinant human fibroblast growth factor 9; PBS, phosphate-buffered saline; TGF-β, transforming growth factor-β; BMP, bone morphogenetic protein; ANOVA, One-way Analysis of Variance; PVDF, Polyvinylidene fluoride; DAB, 3.3-N-Diaminobenzidine Tetrahydrochloride; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RIPA, Radio-Immunoprecipitation Assay.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

No human subjects were involved in this study. The research utilized animals, and all experimental procedures adhered to the Guide for the Care and Use of Laboratory Animals as published by the National Institutes of Health (NIH). This experiment received authorization from the Ethics Committee of China Medical University.

Consent for Publication

All authors approved the final manuscript and the submission to this journal.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors have declared that no competing interests exist.

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