Original Article

Cucurbitacin promotes hair growth in mice by inhibiting the expression of fibroblast growth factor 18

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Background: The inhibition of fibroblast growth factor 18 (FGF18) promotes the transition of hair follicles (HFs) from the telogen phase to the anagen phase. Cucurbitacin has been shown to have a good effect in promoting hair cell growth. This study explored the potential effect of cucurbitacin on hair growth and its effect on FGF18 expression in mice.

Methods: Male C57BL/6J mice were randomly divided into the following two groups: (I) the vehicle group; and (II) the cucurbitacin group. Matrix cream and cucurbitacin cream were applied to the depilated skin on the back of the vehicle group mice and the cucurbitacin group mice, respectively. On days 3, 6, 9, 12, 15, and 18, the hair growth in the depilated dorsal skin of the mice was recorded with a digital camera and a HF detector, and the HF cycle status of the mice was observed by hematoxylin and eosin (H&E) staining. In addition, the level of FGF18 messenger ribonucleic acid (mRNA) in the dorsal skin was measured on days 15 and 18 by quantitative real-time polymerase chain reaction (qRT-PCR), while the level of FGF18 protein was measured by western blot and immunofluorescence staining.

Results: The dorsal skin to which the cucurbitacin cream was applied began to darken on day 6 and grew hairs on day 9, which was 3 days earlier than the dorsal skin to which the matrix cream was applied. The H&E staining revealed a transition from the telogen phase to the anagen phase 3 days earlier for the cucurbitacin cream-treated skin than the matrix cream-treated skin. In addition, the skin treated with cucurbitacin cream also showed a significant decrease in FGF18 mRNA as seen by qRT-PCR, and reduced FGF18 protein levels as detected by western blot and immunofluorescence staining compared to the skin treated with matrix cream only.

Conclusions: Cucurbitacin significantly reduced the levels of FGF18 mRNA and protein in the dorsal skin of mice to accelerate the HFs to enter the anagen phase earlier, thereby promoting the regeneration of hair. Thus, cucurbitacin can be considered a new and valuable agent for the development of anti-hair loss products.

Keywords: Cucurbitacin; hair follicle cycle; fibroblast growth factor 18 (FGF18)

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Introduction

The hair follicle (HF) is the main accessory organ of mammalian skin. It exhibits complex morphological changes and has a complex physiological development process (1). The development of HFs is characterized by cyclic changes, and the growth cycle of HFs determines the growth and shedding of the hairs (2). The HF growth cycle consists of the 3 following distinct phases: the anagen phase, the catagen phase, and the telogen phase (3,4). A HF has different structural characteristics at different phases of the growth cycle. During the anagen phase, the dermal papilla becomes larger and grows from the dermis to the subcutaneous tissue. The hair matrix cells surrounding the dermal papilla undergo terminal differentiation to produce a layer of differentiated keratinocytes that form the inner root sheath and hair shaft (5). The HF gradually reaches its maximum length, and its structure becomes clearly visible and easily recognized. As the supply of hair matrix cells decreases, the HF enters the catagen phase, the lower part of the HF gradually disappears, and the hair matrix separates from the dermal papilla (6). After the HF enters the telogen phase, the dermal papilla gradually becomes compact and spherical. At this stage, the HF does not show the inner root sheath and is completely surrounded by interfollicular dermal fibroblasts. At the end of the telogen phase, the HF will usher in the anagen phase and enter a new round of the HF cycle (7).

Hair loss occurs when the hair growth cycle is disrupted or the HF is mechanically destroyed (8,9). Taking androgenic alopecia (AGA) as an example, the continuous shortening of the anagen phase of the HF and the continuous extension of the telogen phase causes the HF to continue to miniaturize, and it may eventually stay in the telogen phase permanently, leading to alopecia (10). Thus, the key to restoring hair growth is to promote the transition of HFs from the telogen phase to the anagen phase.

It has been suggested that the high expression of dihydrotestosterone (DHT), an androgen, is the main cause of AGA (11,12). By regulating a series of growth factors related to the HF cycle, DHT causes the HF enter the telogen phase in advance, and the HF gradually miniaturizes, eventually shrinking and falling off. The drugs available to treat hair loss mainly include finasteride, minoxidil, and corticosteroids. Finasteride is a 5-alpha reductase type II inhibitor that is effective in inhibiting the production of DHT (13). Minoxidil promotes hair growth by increasing blood flow (14). Corticosteroids are used to treat hair loss caused by the immune system-mediated destruction of the HFs (15). However, the long-term use of these drugs has certain side effects on humans, such as sexual dysfunction, depression, chest pain, dyspnea, myocardial infarction, and diabetes (16-19). Thus, it is urgent to develop safer and more effective drug substitutes. In addition, the psychological state and the change in appearance caused by sparse hair volume seriously affect people's quality of life. With improvements in living standards and an in-depth understanding of alopecia disease, there is also a higher demand for treatments to prevent alopecia and promote hair growth.

The regulation of the HF cycle is mainly controlled by the mesenchymal-epithelial interaction (MEI) of the HF (20). The MEI is mediated by growth factors, including fibroblast growth factors (FGFs) and numerous protein regulators (21). The FGF family consists of 23 members, of which FGF1, FGF2, FGF7, and FGF10 promote the growth of HFs and maintain them in the anagen phase (22-25), FGF9 and FGF13 induce the transition of HFs from the telogen phase to the anagen phase (26,27), and FGF20, FGF21, and FGF22 promote the morphogenesis and localization of the HFs (28-30). In addition to the direct negative regulation of FGF5 in the growth cycle (31), FGF18 is highly expressed in the bulge region of the HFs of mice in the telogen phase, maintaining the HF stem cells in a quiescent state, so that the HFs stay in the telogen phase without entering the anagen phase (32,33). It has been reported that FGF18 can inhibit the growth of HF bulge stem cells *in vitro* (34).

FGF18 is involved in the regulation of the hair follicle cycle during hair growth. FGF18 maintains hair follicle telogen phase and determines its duration, while suppressing anagen phase (32). Forkhead transcription factor 1 (Foxp1) is an important factor in maintaining HFs in the telogen phase, and FGF18 is a key downstream gene of Foxp1. A previous study showed that exogenous FGF18 prevents the premature activation of HF stem cells in Foxp1 null mice, and FGF18 knockout in bulge cells causes hair to enter the anagen phase earlier (35). In a previous study, we found that the topical application of a cream containing cholesterol-modified FGF18-targeting small-interfering ribonucleic acid (siRNA) promotes the early transition of HFs from the telogen phase to the anagen phase and restores hair regeneration (36). The above-mentioned studies showed that FGF18 is a key regulator that keeps HFs in the telogen phase and prevents the activation of HFs and consequently, their entry into the anagen phase. Thus, FGF18 can be

considered a potential target for germinal hair. Inhibiting the expression of FGF18 or antagonizing its activity may promote hair follicles to enter the anagen phase, thereby promoting hair follicle growth.

Many plant extracts have been shown to induce the transition of HFs from the telogen phase to the anagen phase and have unique advantages in promoting hair growth (37-40). Extracts are generally less toxic, less costly, and more readily available than the commonly used anti alopecia drugs on the market (such as finasteride, minoxidil, and corticosteroids). Cucurbitacin and its derivatives are a class of highly oxidized tetracyclic triterpenoids mainly produced in Cucurbitaceae plants (41,42). Cucurbitacin has a wide range of pharmacological effects, such as anti-inflammatory, antiviral, anti-malarial, anti-tumor (43), antipyretic, analgesic, and hepatoprotective effects (44-46). At present, drugs with cucurbitacin as the main ingredient have been developed into tablets or capsules for the treatment of hepatitis and liver cancer. However, there is no cucurbitacin cream that can be applied in clinic. Studies have shown that cucurbitacin has great potential in the treatment of psoriasis, anti-cutaneous T-cell lymphoma, anti-melanoma, and other skin diseases (47-50). Further, it has been found that cucurbitacin significantly promotes the growth of hair cells cultured *in vitro*, and its effect is 1,000 times stronger than that of minoxidil (51). Thus, we hypothesized that the application of cucurbitacin may promote the growth of hair cells and the transition of the HF cycle from the telogen phase to the anagen phase in mice, thus inducing hair regeneration.

In this study, a mouse model established by depilation was used to evaluate the role of cucurbitacin in the regulation of the HF cycle in mice and the effect of cucurbitacin on the expression of FGF18, and to explore the potential mechanism by which cucurbitacin might affect hair growth in mice. We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-4423/rc).

Methods

Regents

Cucurbitacin was purchased from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China).

Cream preparation

To prepare the matrix cream for topical application, 2.0 g of 2-ethylhexyl palmitate was first mixed with 0.25 g of azone and dissolved to achieve an oil phase. Next, 1.0 g of propylene glycol and 0.25 g of benzethonium chloride were mixed with 45.0 g of water and dissolved to achieve an aqueous phase. After that, 1.5 g of emulsifier was added to the oil phase and stirred slowly to form a uniform mixture. Finally, the aqueous phase was slowly added to the uniform mixture in stages, and the mixture was stirred slowly in the same direction to form the matrix cream. To prepare the cream containing cucurbitacin, 330 μg of cucurbitacin was mixed with 2.0 g of 2-ethylhexyl palmitate and dissolved in 0.25 g of azone during the preparation of the oil phase. The rest of the steps were essentially the same as those described above.

Mice and housing

A total of 20 healthy male C57BL/6J mice (7-weeks old, 18–22 g) were purchased from GemPharmatech Co., Ltd. (Jiangsu, China) (No. SCXK-2018-0008). All the mice were housed at 20±2 ℃ in the presence of 50–55% relative humidity and placed on a 12-h light and dark cycle. The mice were fed with a standard laboratory diet and had *ad libitum* access to food and water at the Wenzhou Medical University. The experiments were performed under a project license [No. SYXK(Zhe) 2020-0014] granted by the Ethics Committee of Wenzhou Medical University and in compliance with institutional guidelines for the care and use of animals. A protocol was prepared before the study but was not registered.

Mice model and treatments

After the mice were anesthetized with a small animal inhalation anesthesia machine (RWD, China), the hairs on their back skin were removed by shaving with an electric shaver to an area of about 2 cm × 3 cm. Next, a depilatory cream was applied to the shaven region, and 3 minutes later, the depilatory cream was gently wiped off with a piece of gauze dipped in warm water to completely remove the surface hair. The date of shaving was recorded as day 0, and the regrowth of hair on the shaven skin was monitored for 18 consecutive days. The animals were then randomly

divided into the following two groups: (I) the vehicle group, to which the matrix cream was applied; and (II) the cucurbitacin group, to which the cucurbitacin cream was applied twice a day for several consecutive days until the skin tissue was collected. Each group comprised 10 mice. From the 3rd day onward, the back of each animal was photographed every 3 days with a digital camera and a HF detector (CBS, China) to record the growth of the skin hair. Additionally, a mouse in each group was randomly selected to collect skin tissue samples, which were fixed in 4% paraformaldehyde solution for the histological analysis. On days 15 and 18, 3 mice in each group were randomly selected to collect skin samples. The skin specimen from each animal was divided into 3 sections, of which, 1 was used for the histological analysis, and 2 were stored in 2 separate frozen tubes at –80 ℃ for quantitative real-time PCR (qRT-PCR) and western blotting.

H&E staining

The skin tissue samples were first fixed in a 4% paraformaldehyde solution. Next, they were dehydrated in ethanol with increasing concentrations (70%, 80%, 95%, and 100%). After that, they were embedded in paraffin and cut into 7 μm-thick slices. Finally, the slices were stained with hematoxylin and eosin (H&E; Solarbio, Beijing, China) in accordance with the manufacturer's instruction and then observed under a Leica Microsystem (Wetzlar, Germany) to assess the histological changes.

Immunofluorescence staining analysis

FGF18 expression in the HFs was detected by immunofluorescence, and the skin tissue sections were deparaffinized, rehydrated, and incubated in 3% hydrogen peroxide (H_2O_2)/methanol solution for 25 min. Subsequently, the skin tissue antigens were repaired by the high-pressure thermal repair method using sodium citrate buffer. After blocking with 3% bovine serum albumin (BSA) for 4 h, the tissue slices were incubated with an anti-FGF18 antibody (1:500 dilution; Proteintech, Wuhan, China) at 4 ℃ overnight. This was followed by incubation with a secondary antibody at 37 ℃ for 4 h. After that, the tissue slices were washed with phosphate buffered solution and stained with ProLong Gold Antifade reagent containing DAPI (4, 6-diamino-2-phenyl indole) (Life Technologies Corporation, NY, United States), and finally mounted on glass slides and examined under a laser confocal microscope (Olympus, Tokyo, Japan).

qRT-PCR analysis

Total RNA was isolated from the skin tissue samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed with a PrimeScript RT reagent Kit (Takara, Dalian, China) using the isolated RNA as a template. SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) was used for the qRT-PCR, which was performed with a LC96 system (Roche, Basel, Switzerland). The following primers were used: FGF18 (forward, 5'-GGAGCAGGTGACCTTTGATGAG-3'; reverse, 5'-GAGAGGTGCCAGTTGATGATGG-3'); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward, 5'-AGAAGGTGGTGAAGCAGGCATC-3'; reverse, 5'-CGAAGGTGGAAGAGTGGGAGTTG-3'). The relative messenger RNA (mRNA) level of FGF18 was quantified using the $2^{-\Delta\Delta Ct}$ method and normalized to the level of GAPDH.

Western blotting

Total protein was extracted from the skin tissue samples using radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Jiangsu, China) containing protease inhibitors and phosphatase inhibitors. The protein concentration of the extract was determined with a bicinchoninic acid assay kit (Beyotime, Jiangsu, China). After that, an equivalent amount of protein (30 μg) from each skin tissue sample was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the protein bands in the gel were then transferred to a polyvinylidene fluoride (PVDF) membrane. Subsequently, the membrane was incubated in 5% BSA for 2 h followed by incubation with anti-FGF18 (Proteintech, Chicago, IL, United States) or anti-Tubulin (Cell Signaling Technology, Beverly, MA, United States) antibodies at 4°C for overnight. The blot was then washed with Tris-buffered saline with Tween-20 (TBST) and incubated with goat anti-rabbit antibody (Cell Signaling Technology, Beverly, MA, USA) at room temperature for 1.5 h. Finally, the blot was detected with a chemiluminescence substrate (Thermo scientific, NY, the United States), and images of the blot were captured with an Amersham Imager (GE Healthcare Biosciences, Pittsburgh, PA, the United States).

Statistical analysis

All the experiment data were analyzed using GraphPad Prism 6.0 software (GraphPad, San Diego, CA, United States). A comparison of the 2 sets of data was performed by a multiple *t*-tests analysis. All the quantitative data were expressed as the mean ± standard error of mean (SEM) from at least 3 independent experiments. Statistical significance was considered to have been reached at the $P<0.05$ or $P<0.01$ levels.

Results

Application of cucurbitacin promotes hair regeneration in depilated skin

A mouse depilation model was used to evaluate the effect of cucurbitacin on hair growth. *Figure 1A* shows the experimental design for the treatment of mice in this model. The date on which the hairs on the back skin of the animals were shaven was recorded as day 0. On day 1 after shaving, matrix cream or cucurbitacin cream was topically applied to the shaven region of the skin twice daily until the skin tissue was collected. Specimens of the dorsal skin were collected on days 3, 6, 9, 12, 15, and 18 for H&E staining or RNA and protein extraction. To observe the changes in hair growth, gross observation of the skin was performed (see *Figure 1B*). On day 6, black patches appeared on the skin to which the cucurbitacin cream had been applied, indicating that the HFs in the skin had begun the transition from the telogen phase to the anagen phase. Short hairs started to grow on day 9, and the hairs grew extensively from day 12 and became very thick on day 18. Conversely, the skin to which the matrix cream was applied remained pink on day 6, and black spots did not appear until day 9. Little hair growth was seen on day 12, and hair growth was significantly more abundant on day 15 but then became thin on day 18.

Similarly, photographs taken by the HF detector showed that the skin to which the cucurbitacin cream was applied turned dark on day 6, grew hair on day 9, and the hair became very thick on day 18. The skin to which the matrix cream was applied began to darken on day 9, and significant hair was observed only on day 15 (see *Figure 1C*). Hair growth and skin darkness were more significant in the skin to which the cucurbitacin cream had been applied than in the skin to which matrix cream had been applied.

The morphological changes of the HFs between the cucurbitacin cream-treated and matrix cream-treated skin were compared. The hair bulbs in both groups were located in the superficial dermis on day 3, but they were miniaturized and constricted, which indicated that the HFs were in the telogen phase (see *Figure 1D*). From day 6 onward, the hair bulbs of the cucurbitacin cream-treated skin began to become plump and migrate downward, indicating that the HFs were in a transition from the telogen phase to the anagen phase. On day 9, the hair bulbs were located at the dermal-subcutaneous junction, indicating that the HFs were in the anagen phase. By day 15, the hair shaft surrounded by the hair canal emerged through the epidermis. Conversely, the HFs in the matrix cream-treated skin showed a telogen-to-anagen transition only from day 9 onward, with hair bulbs localized at the dermis-subcutaneous junction from day 12 onward. These results showed that the application of cucurbitacin cream to the depilated back skin of mice effectively shortened the telogen phase of HFs by 3 days and promoted the entry of HFs into the anagen phase.

Cucurbitacin decreases the expression of FGF18 in mice dorsal skin

To further investigate how cucurbitacin might promote the HFs to enter the anagen phase, the expression of FGF18 in the depilated back skin of the mice was determined following the application of the matrix cream or cucurbitacin cream. The level of FGF18 mRNA in the skin to which the cucurbitacin cream had been applied was significantly lower than that in the skin to which the matrix cream had been applied on days 15 and 18 as revealed by the qRT-PCR, and the degree of reduction was independent of the time of application of the cucurbitacin cream (see *Figure 2A*). Similarly, the level of FGF18 protein in the skin to which the cucurbitacin cream had been applied was also reduced by about 2-fold compared to that of the skin to which the matrix cream had been applied, and the reduction was significant, as revealed by western blot (see *Figure 2B*).

On days 15 and 18, FGF18 was expressed in the epidermis and hair root sheath of the skin to which the matrix cream had been applied, while the level of FGF18 was significantly reduced in the hair root sheath for the cucurbitacin-treated skin as shown by the immunofluorescence staining of the skin (see *Figure 2C*). These results clearly indicated that the topical application of cucurbitacin cream decreased the expression of FGF18, both at the mRNA and protein levels, effectively promoting hair growth.

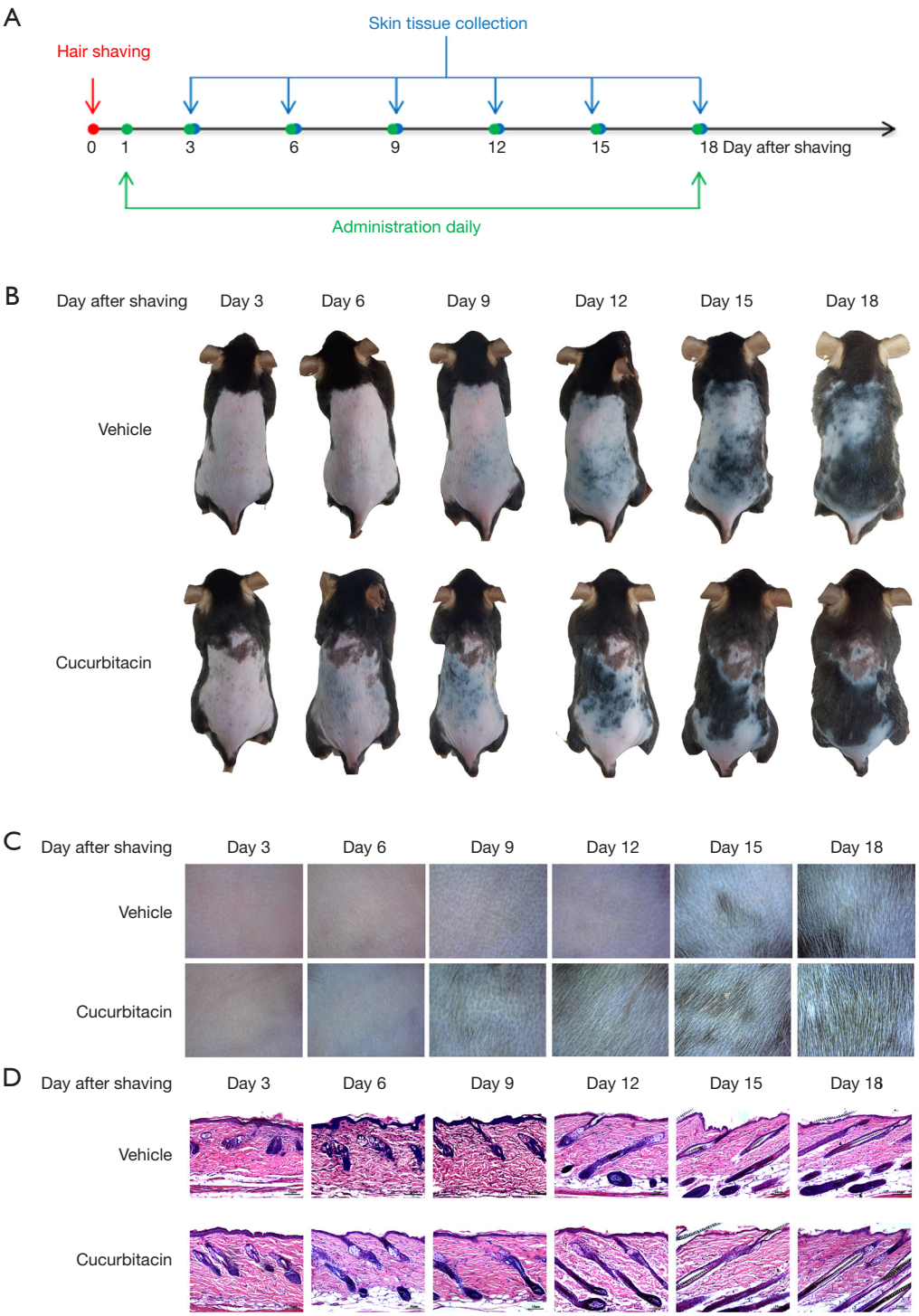

Figure 1 Effect of cucurbitacin on hair regeneration in the depilated back skin of mice. (A) Diagram depicting the experimental design. (B) Gross observation images of the depilated back skin following the application of matrix cream or cucurbitacin cream. The images were taken on different days as indicated. (C) Changes in the morphology of the HFs in the depilated skin after the application of matrix cream or cucurbitacin cream. The images were taken with a HF detector at 200× magnification and on different days as indicated. (D) H&E staining of dorsal skin tissues after the application of matrix cream or cucurbitacin cream. Images were taken on different days as indicated. Scale bars: 10 μm. HF, hair follicle; H&E, hematoxylin and eosin.

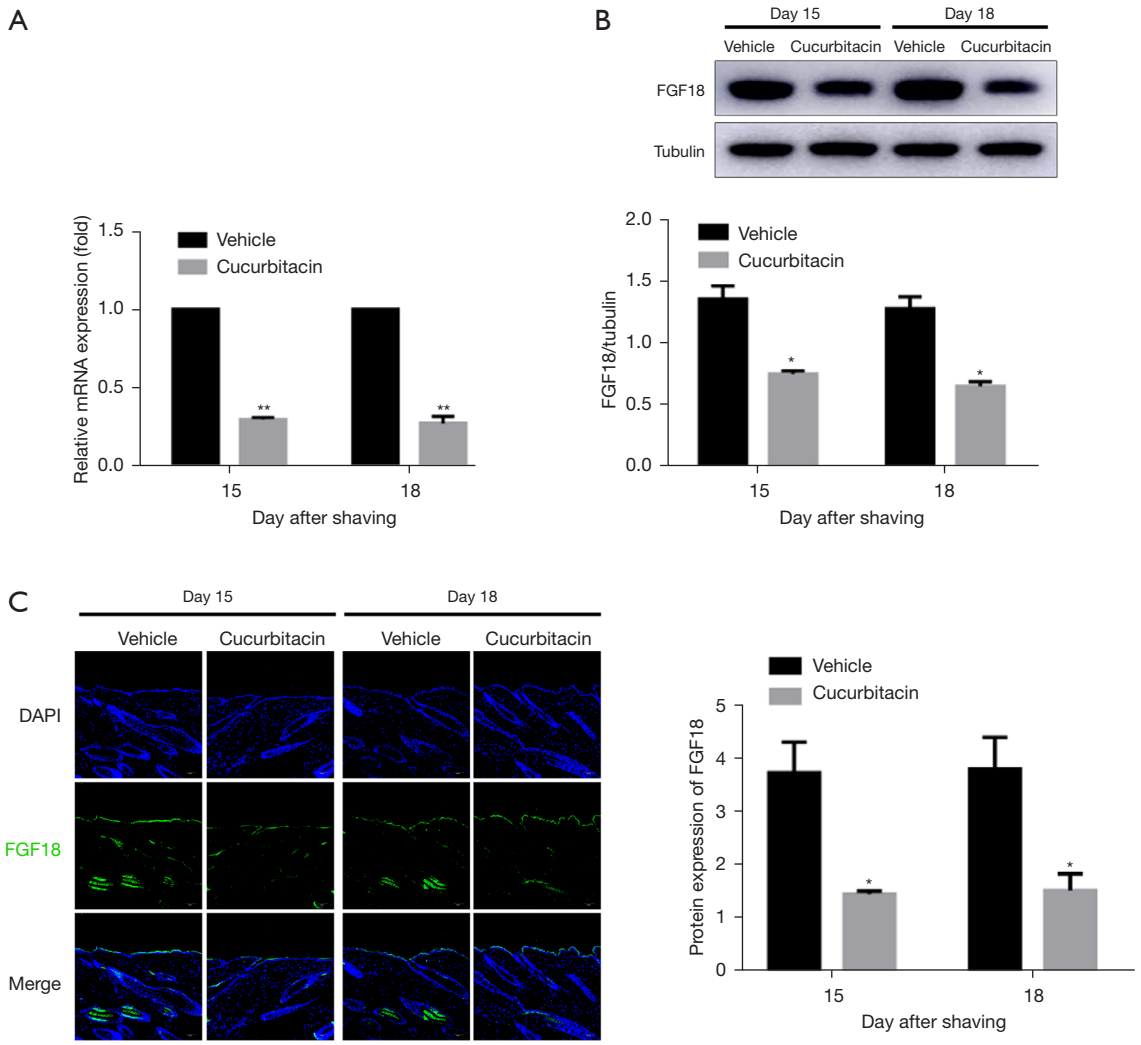

Figure 2 Effect of cucurbitacin on the expression of FGF18 in the depilated back skin of mice. (A) qRT-PCR analysis of FGF18 mRNA expression levels in the skin on days 15 and 18 after applying matrix cream or cucurbitacin cream. (B) Western blot analysis of FGF18 protein expression levels in the skin on days 15 and 18 after applying matrix cream or cucurbitacin cream. The plot below the blot shows the quantitation of the FGF18 protein in the blot as determined by grayscale densitometry. The results were normalized to α-tubulin expression. (C) Immunofluorescence staining of FGF18 protein expression levels in the skin on days 15 and 18 after applying matrix cream or cucurbitacin cream. Green fluorescence represents FGF18 protein. All tissue slices were counterstained with DAPI (blue). Scale bars: 50 μm. The plot besides the image compares the level of FGF18 protein between the two different treatments. Data in the plots are mean ± SEM from 3 experiments, '*' and '**' indicate that the results of the mice that received the cucurbitacin cream treatment differed significantly to the results of the mice that received the matrix cream treatment at the $P<0.05$ and $P<0.01$ levels, respectively. FGF18, fibroblast growth factor 18; qRT-PCR, quantitative real-time polymerase chain reaction; DAPI, 4, 6-diamino-2-phenyl indole; SEM, standard error of mean.

Discussion

The HF is the main structure that grows hair, and a normal HF cycle is key to ensuring normal hair growth. Hair loss occurs when the HFs stay in the telogen phase due to interference with the HF cycle. Thus, the mobilization of HFs from the telogen phase to the anagen phase is an important factor in promoting hair regeneration.

Cucurbitacin, which is extracted from Cucurbitaceae plants, has a wide range of pharmacological effects and potential for the treatment of skin diseases. A previous

study suggested that the hair growth-promoting effect of cucurbitacin is very significant (51). However, whether and how cucurbitacin can play a role in promoting hair growth in animal skin has not been studied before. In this study, we provided some insights into the hair growth-promoting mechanism of cucurbitacin and the cucurbitacin-mediated regulation of the HF cycle in mice.

At present, cucurbitacin is mainly used in two ways: oral and injection. However, the commercially available cucurbitacin tablets or capsules limit their clinical efficacy due to gastrointestinal side effects and non-specific toxicity (52). It also faces the problem of low oral bioavailability when applied to experimental animals by gavage (53). The way of intraperitoneal injection is related to the defect of patients' compliance and acceptance (54). Considering that cream has the advantages of easy preparation, high drug loading and painlessness, we prepared cucurbitacin cream for subsequent administration to mice. Due to the large molecular weight of cucurbitacin, we chose a matrix cream composed of an oil phase, a water phase, and an emulsifier to improve the solubility and skin permeability of cucurbitacin (51). Depilation was used to synchronize the HF growth cycle on the back skin of mice before the application of the cucurbitacin cream. During the telogen phase, the HF is in a dormant state, and no hair is located on the surface of the skin. The melanocyte stem cells in the skin are also in a quiescent state (55), and thus the depilated skin has a pinkish color. After the HF has entered the anagen phase, the hair matrix cells proliferate rapidly, differentiate into the hair shaft and the inner root sheath, and the hair is located on the skin surface through the epidermis (56). Melanocyte stem cells are simultaneously activated, and the melanocytes present in the hair matrix produce melanin granules and transfer them to keratinocytes, at which time dark patches appear on the depilated skin (57). Based on such principles, we were able to determine the effect of cucurbitacin on hair growth on the depilated skin of the mice from the time that elapsed between the application of the cucurbitacin cream and the appearance of dark patches on the skin and by observing the hair growth status. Cucurbitacin had a good effect on hair growth, as evidenced by the earlier appearance of black patches on the skin and thicker hair (see *Figure 1B*,*1C*) compared to the matrix cream-only treatment.

HFs are micro organs formed by the interaction of epidermis and dermis (58). The HF has different structural characteristics at various stages of the growth cycle. In the telogen phase, the dermal papilla atrophy and the HF are completely surrounded by dermal fibroblasts. After the HF enters the anagen phase, the dermal papilla becomes larger, and as the secondary hair germ cells descend into the dermis, a new HF is formed in the deep dermis (59). During growth, the hair matrix cells located within the hair bulb rapidly proliferate to give rise to the hair shaft, and the tip of the hair shaft contained in the hair tube crosses the epidermis to reach the skin surface. Thus, the morphological and structural changes of the HF can be observed by H&E staining, and the stage of the HF cycle can be judged. Our H&E results showed that the HFs in the skin to which the cucurbitacin cream had been applied entered the anagen phase (from the telogen phase) 3 days earlier than the HFs in the skin to which the matrix cream had been applied (see *Figure 1D*), indicating that cucurbitacin activated the HFs and promoted hair regeneration by shortening the telogen phase while extending the anagen phase of the HF growth cycle.

Hair growth is a complex process involving a variety of regulatory signaling molecules, and fibroblast growth factors play an important role in HF morphogenesis and the HF cycle. FGF18 is mainly expressed in the inner root sheath in the anagen phase and the HF bulge in the telogen phase, and its expression level increases in the telogen phase and decreases in the anagen phase (60). In FGF18 conditional knockout mice in which the FGF18 gene in keratin 5-positive epithelial cells was selectively knocked out, the telogen phase of the HF was significantly shortened, resulting in a strikingly rapid succession of hair cycles, thus proving that the loss of FGF18 signal significantly accelerates the initiation of the anagen phase (32). However, in wild-type mice, a local intradermal injection of FGF18 protein was found to strongly inhibit HF growth during the HF anagen phase (32). These studies showed that FGF18 is a key maintenance factor of the telogen phase and inhibiting the expression of FGF18 or antagonizing its activity promotes HFs to enter the anagen phase, which in turn promotes HF growth. Notably, the application of the cucurbitacin cream caused a significant drop in the FGF18 mRNA and protein levels compared to the application of the matrix cream (see *Figure 2*), providing clear evidence that cucurbitacin plays a role in promoting the anagen phase of the HF and the activation of the HF by inhibiting FGF18 expression.

We investigated the effects of cucurbitacin on hair growth and the expression of FGF18 in mice; however, we still do not know through which signaling pathway cucurbitacin effectively regulates the expression of FGF18

to regulate the HF growth cycle. Thus, our follow-up research will focus on the signaling pathway by which cucurbitacin regulates the HF growth cycle and the effect of cucurbitacin on patients with hair loss.

Conclusions

The application of cucurbitacin cream significantly downregulated the mRNA and protein levels of FGF18 in the back skin of mice. Cucurbitacin was also shown to promote the transition of the HFs in the skin, from the telogen phase to the anagen phase, effectively promoting hair growth. These findings confirm the role of cucurbitacin in promoting the growth of hair cells, which is of great significance for the research and development of new anti-hair loss products.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-4423/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-4423/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-4423/coif). All authors report that this work was supported by Infinitus (China) Co., Ltd. The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The experiments were performed under a project license [No. SYXK(Zhe) 2020-0014] granted by the Ethics Committee of Wenzhou Medical University and in compliance with institutional guidelines for the care and use of animals.

References

1. Schneider MR, Schmidt-Ullrich R, Paus R. The hair follicle as a dynamic miniorgan. Curr Biol 2009;19:R132-42.
2. Choi BY. Targeting Wnt/β-Catenin Pathway for Developing Therapies for Hair Loss. Int J Mol Sci 2020;21:4915.
3. Müller-Röver S, Handjiski B, van der Veen C, et al. A comprehensive guide for the accurate classification of murine hair follicles in distinct hair cycle stages. J Invest Dermatol 2001;117:3-15.
4. Bernard BA. The hair follicle enigma. Exp Dermatol 2017;26:472-7.
5. Alonso L, Fuchs E. The hair cycle. J Cell Sci 2006;119:391-3.
6. Blanpain C, Fuchs E. Epidermal stem cells of the skin. Annu Rev Cell Dev Biol 2006;22:339-73.
7. Houschyar KS, Borrelli MR, Tapking C, et al. Molecular Mechanisms of Hair Growth and Regeneration: Current Understanding and Novel Paradigms. Dermatology 2020;236:271-80.
8. Paus R, Cotsarelis G. The biology of hair follicles. N Engl J Med 1999;341:491-7.
9. Stenn KS, Paus R. Controls of hair follicle cycling. Physiol Rev 2001;81:449-94.
10. Falto-Aizpurua L, Choudhary S, Tosti A. Emerging treatments in alopecia. Expert Opin Emerg Drugs 2014;19:545-56.
11. Schweikert HU, Wilson JD. Regulation of human hair growth by steroid hormones. II. Androstenedione metabolism in isolated hairs. J Clin Endocrinol Metab 1974;39:1012-9.
12. Kaufman KD. Androgens and alopecia. Mol Cell Endocrinol 2002;198:89-95.
13. Chaudhary UB, Turner JS. Finasteride. Expert Opin Drug

Metab Toxicol 2010;6:873-81.
14. Otomo S. Hair growth effect of minoxidil. Nihon Yakurigaku Zasshi 2002;119:167-74.
15. Garg S, Messenger AG. Alopecia areata: evidence-based treatments. Semin Cutan Med Surg 2009;28:15-8.
16. La Vignera S, Aversa A, Cannarella R, et al. Pharmacological treatment of lower urinary tract symptoms in benign prostatic hyperplasia: consequences on sexual function and possible endocrine effects. Expert Opin Pharmacother 2021;22:179-89.
17. Nguyen DD, Marchese M, Cone EB, et al. Investigation of Suicidality and Psychological Adverse Events in Patients Treated With Finasteride. JAMA Dermatol 2021;157:35-42.
18. Beach RA, McDonald KA, Barrett BM, et al. Side effects of low-dose oral minoxidil for treating alopecia. J Am Acad Dermatol 2021;84:e239-e40.
19. Mbengue M, Goumri N, Niang A. IgG4-related kidney disease: Pathogenesis, diagnosis, and treatment. Clin Nephrol 2021;95:292-302.
20. Chuong CM, Cotsarelis G, Stenn K. Defining hair follicles in the age of stem cell bioengineering. J Invest Dermatol 2007;127:2098-100.
21. Jacques BE, Dabdoub A, Kelley MW. Fgf signaling regulates development and transdifferentiation of hair cells and supporting cells in the basilar papilla. Hear Res 2012;289:27-39.
22. Lin WH, Xiang LJ, Shi HX, et al. Fibroblast growth factors stimulate hair growth through β-catenin and Shh expression in C57BL/6 mice. Biomed Res Int 2015;2015:730139.
23. Danilenko DM, Ring BD, Yanagihara D, et al. Keratinocyte growth factor is an important endogenous mediator of hair follicle growth, development, and differentiation. Normalization of the nu/nu follicular differentiation defect and amelioration of chemotherapy-induced alopecia. Am J Pathol 1995;147:145-54.
24. Igarashi M, Finch PW, Aaronson SA. Characterization of recombinant human fibroblast growth factor (FGF)-10 reveals functional similarities with keratinocyte growth factor (FGF-7). J Biol Chem 1998;273:13230-5.
25. Itoh N. FGF10: A multifunctional mesenchymal-epithelial signaling growth factor in development, health, and disease. Cytokine Growth Factor Rev 2016;28:63-9.
26. Gay D, Kwon O, Zhang Z, et al. Fgf9 from dermal γδ T cells induces hair follicle neogenesis after wounding. Nat Med 2013;19:916-23.
27. Kawano M, Suzuki S, Suzuki M, et al. Bulge- and basal layer-specific expression of fibroblast growth factor-13 (FHF-2) in mouse skin. J Invest Dermatol 2004;122:1084-90.
28. Huh SH, Jones J, Warchol ME, et al. Differentiation of the lateral compartment of the cochlea requires a temporally restricted FGF20 signal. PLoS Biol 2012;10:e1001231.
29. Liu X, Zhang P, Zhang X, et al. Fgf21 knockout mice generated using CRISPR/Cas9 reveal genetic alterations that may affect hair growth. Gene 2020;733:144242.
30. Li C, Guo H, Xu X, et al. Fibroblast growth factor receptor 2 (Fgfr2) plays an important role in eyelid and skin formation and patterning. Dev Dyn 2001;222:471-83.
31. Suzuki S, Ota Y, Ozawa K, et al. Dual-mode regulation of hair growth cycle by two Fgf-5 gene products. J Invest Dermatol 2000;114:456-63.
32. Kimura-Ueki M, Oda Y, Oki J, et al. Hair cycle resting phase is regulated by cyclic epithelial FGF18 signaling. J Invest Dermatol 2012;132:1338-45.
33. Hsu YC, Pasolli HA, Fuchs E. Dynamics between stem cells, niche, and progeny in the hair follicle. Cell 2011;144:92-105.
34. Blanpain C, Lowry WE, Geoghegan A, et al. Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. Cell 2004;118:635-48.
35. Leishman E, Howard JM, Garcia GE, et al. Foxp1 maintains hair follicle stem cell quiescence through regulation of Fgf18. Development 2013;140:3809-18.
36. Zhao J, Lin H, Wang L, et al. Suppression of FGF5 and FGF18 Expression by Cholesterol-Modified siRNAs Promotes Hair Growth in Mice. Front Pharmacol 2021;12:666860.
37. Zhang Y, Ni C, Huang Y, et al. Hair Growth-Promoting Effect of Resveratrol in Mice, Human Hair Follicles and Dermal Papilla Cells. Clin Cosmet Investig Dermatol 2021;14:1805-14.
38. Truong VL, Jeong WS. Hair Growth-Promoting Mechanisms of Red Ginseng Extract through Stimulating Dermal Papilla Cell Proliferation and Enhancing Skin Health. Prev Nutr Food Sci 2021;26:275-84.
39. Hyun J, Im J, Kim SW, et al. Morus alba Root Extract Induces the Anagen Phase in the Human Hair Follicle Dermal Papilla Cells. Pharmaceutics 2021;13:1155.
40. Bhinge SD, Bhutkar MA, Randive DS, et al. Screening of hair growth promoting activity of Punica granatum L. (pomegranate) leaves extracts and its potential to exhibit antidandruff and anti-lice effect. Heliyon 2021;7:e06903.
41. Duncan KL, Duncan MD, Alley MC, et al. Cucurbitacin

E-induced disruption of the actin and vimentin cytoskeleton in prostate carcinoma cells. Biochem Pharmacol 1996;52:1553-60.
42. Kaushik U, Aeri V, Mir SR. Cucurbitacins - An insight into medicinal leads from nature. Pharmacogn Rev 2015;9:12-8.
43. Lin X, Farooqi AA. Cucurbitacin mediated regulation of deregulated oncogenic signaling cascades and non-coding RNAs in different cancers: Spotlight on JAK/STAT, Wnt/β-catenin, mTOR, TRAIL-mediated pathways. Semin Cancer Biol 2021;73:302-9.
44. Jayaprakasam B, Seeram NP, Nair MG. Anticancer and antiinflammatory activities of cucurbitacins from Cucurbita andreana. Cancer Lett 2003;189:11-6.
45. Chen JC, Chiu MH, Nie RL, et al. Cucurbitacins and cucurbitane glycosides: structures and biological activities. Nat Prod Rep 2005;22:386-99.
46. Salehi B, Capanoglu E, Adrar N, et al. Cucurbits Plants: A Key Emphasis to Its Pharmacological Potential. Molecules 2019;24:1854.
47. Li ZJ, Shin JM, Choi DK, et al. Inhibitory effect of cucurbitacin B on imiquimod-induced skin inflammation. Biochem Biophys Res Commun 2015;459:673-8.
48. van Kester MS, Out-Luiting JJ, von dem Borne PA, et al. Cucurbitacin I inhibits Stat3 and induces apoptosis in Sézary cells. J Invest Dermatol 2008;128:1691-5.
49. Liu WB, Wang HL, Chen L, et al. Cucurbitacin E inhibits cellular proliferation and induces apoptosis in melanoma by suppressing HSDL2 expression. Chin Med 2022;17:28.
50. Nguyen BC, Be Tu PT, Tawata S, et al. Combination of immunoprecipitation (IP)-ATP_Glo kinase assay and melanogenesis for the assessment of potent and safe PAK1-blockers in cell culture. Drug Discov Ther 2015;9:289-95.
51. Nguyen BC, Taira N, Maruta H, et al. Artepillin C and Other Herbal PAK1-blockers: Effects on Hair Cell Proliferation and Related PAK1-dependent Biological Function in Cell Culture. Phytother Res 2016;30:120-7.
52. Bartalis J, Halaweish FT. Relationship between cucurbitacins reversed-phase high-performance liquid chromatography hydrophobicity index and basal cytotoxicity on HepG2 cells. J Chromatogr B Analyt Technol Biomed Life Sci 2005;818:159-66.
53. Hunsakunachai N, Nuengchamnong N, Jiratchariyakul W, et al. Pharmacokinetics of cucurbitacin B from Trichosanthes cucumerina L. in rats. BMC Complement Altern Med 2019;19:157.
54. Lv Q, Shen C, Li X, et al. Mucoadhesive buccal films containing phospholipid-bile salts-mixed micelles as an effective carrier for Cucurbitacin B delivery. Drug Deliv 2015;22:351-8.
55. Slominski A, Wortsman J, Plonka PM, et al. Hair follicle pigmentation. J Invest Dermatol 2005;124:13-21.
56. Cotsarelis G. Epithelial stem cells: a folliculocentric view. J Invest Dermatol 2006;126:1459-68.
57. Lee KH, Choi D, Jeong SI, et al. Eclipta prostrata promotes the induction of anagen, sustains the anagen phase through regulation of FGF-7 and FGF-5. Pharm Biol 2019;57:105-11.
58. Ji S, Zhu Z, Sun X, et al. Functional hair follicle regeneration: an updated review. Signal Transduct Target Ther 2021;6:66.
59. Hardy MH. The secret life of the hair follicle. Trends Genet 1992;8:55-61.
60. Kawano M, Komi-Kuramochi A, Asada M, et al. Comprehensive analysis of FGF and FGFR expression in skin: FGF18 is highly expressed in hair follicles and capable of inducing anagen from telogen stage hair follicles. J Invest Dermatol 2005;124:877-85.

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