



ORIGINAL ARTICLE

Expression Pattern and Role of Klotho in Human Hair Follicles

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Background: Klotho protein plays a pivotal role in aging regulation. However, it is unclear whether klotho is expressed in human hair follicles and is correlated with hair growth. **Objective:** The purpose of this study was to determine the expression pattern and role of klotho in human hair follicles. **Methods:** We examined the klotho expression patterns in human hair follicles from young and aged donors. Furthermore, we examined the functional roles of klotho on human hair growth using klotho siRNA and klotho recombinant protein. **Results:** Interestingly, klotho was expressed in human hair follicles at both gene and protein levels. In hair follicles, prominent klotho expression was mainly observed in the outermost regions of the outer root sheath and hair bulb matrix cells. Quantification of klotho protein expression in young and aged donors showed that klotho expression decreased with aging. In human hair follicle organ culture, klotho silencing promoted premature catagen induction and inhibited human hair growth. Otherwise, klotho protein prolonged human hair growth. **Conclusion:** These results indicate that klotho might be an important regulatory factor for human hair growth and hair cycle change. (Ann Dermatol 31(5) 511~517, 2019)

-Keywords-

Hair growth, Human hair follicle, Klotho

INTRODUCTION

Human hair growth cyclically undergoes three distinct stages of active growth (anagen), regression (catagen) and resting (telogen)¹. Multiple signaling pathways and genes are involved in regulating the hair growth and cycling²⁻⁵. *Klotho*, named after an ancient Greek goddess of fate, is a putative aging suppressor gene⁶, increases the life span when it is over-expressed but hastens aging when it is disrupted in mice. The *klotho* gene with a size of over 50 kb is present on the chromosome 13q12 and contains 5 exons and 4 introns^{7,8}. Alternative splicing of the *klotho* RNA gives rise to two portions: one secreted form and another transmembrane form of klotho protein⁹. The klotho protein plays a crucial role in suppressing aging through modulating various biological signaling pathways. Klotho binds to a cell-surface receptor and inhibits the intracellular signaling of insulin-like growth factor-1¹⁰. Klotho protein has been reported to bind to multiple Wnt ligands and to repress their ability to activate Wnt signaling¹¹. Doi et al.¹² reported that a novel function of secreted klotho is to bind the type I transforming growth factor-beta (TGF- β) receptor and inhibit TGF- β 1 binding to cell surface receptors, thereby inhibiting TGF- β 1 signaling. Klotho is predominantly existed in the kidney. It is also detectable in the prostate, placenta, pancreas, skeletal muscle, testis, ovary, inner ear and small intestine^{10,13}. Defect in *klotho* gene expression in mice results in phenotypes resembling human premature or accelerated aging syndrome⁶. Particularly, skin atrophy and hair loss have been observed as consequences of these mutations. These indicate that klotho

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tho may act as a modulator in the hair growth. Klotho expression has only been detected in a few human tissues and cell lines. However, it is unclear whether klotho is expressed in human hair follicles and is associated with hair growth. The aim of this study was to evaluate the expression pattern and role of klotho in human hair follicles.

MATERIALS AND METHODS

Isolation and culture of human hair follicles

This study was approved by the Institutional Ethics Committee of the Yonsei University Wonju College of Medicine (IRB no. YWMR-13-6-053), and all subjects provided were written informed consents. Scalp samples were taken from the occipital areas of healthy subjects who had not prior or current scalp diseases. Hair follicles were separated and cultured as described previously¹⁴. Each of the anagen hair follicles was carefully transferred to a 24-well plate (Corning, New York, NY, USA) containing 500 μ l of William's E medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10 μ g/ml insulin (Sigma-Aldrich Inc., St. Louis, MO, USA), 10 ng/ml hydrocortisone (Sigma-Aldrich Inc.), 2 mM L-glutamine (Gibco BRL), 100 IU/ml penicillin and 100 μ g/ml streptomycin (Gibco BRL). Test groups were treated with or klotho siRNA (Invitrogen, Carlsbad, CA, USA) or serial dose of klotho protein (R&D Systems, Minneapolis, MN, USA).

Klotho knockdown

Human anagen hair follicles were transfected with *klotho* siRNA (Invitrogen) or control siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using transfection reagent (Santa Cruz Biotechnology) at day 0. After transfection, hair follicles were maintained in a 24-well plate with 500 μ l William's E medium (Biochrom, Cambridge, UK) supplemented with 10 μ g/ml insulin (Sigma-Aldrich Inc.), 10 ng/ml hydrocortisone (Sigma-Aldrich Inc.), 2 mM L-glutamine (Gibco BRL), 100 IU/ml penicillin and 100 μ g/ml streptomycin (Gibco BRL). The efficiency of the blockage was evaluated by reverse transcription polymerase chain reaction (RT-PCR) and western blot analysis.

Measurement of hair length and morphology

The length of hair was calculated as the entire length from the hair bulb base to the hair shaft tip using calculating scales attached to the objective lens in the light microscope (Olympus, Tokyo, Japan). Measurements were done at two day intervals until the 8th day of culture and then were statistically evaluated. During measurements, morphology of hair follicle (anagen, early, mid, and late cata-

gen) was seen and the hair cycle score was analyzed as described in previous reports^{15,16}. For statistical analysis, hair follicles in anagen VI were referred to a score of 100, early catagen hair follicles as a score of 200, mid catagen hair follicles as a score of 300, and late catagen hair follicles as a score of 400.

RT-PCR analysis

Total RNA was collected and RNA concentration was determined using ultraviolet spectrometer at 260 nm. Aliquots (1.0 μ g) of RNA were incubated at 57°C for 10 minutes with ribonuclease free water. After cooling on ice, primer extension and cDNA synthesis was performed with 1X RT-buffer, 5 mM MgCl₂ solution, 1 mM deoxynucleotide triphosphates, 2.5 μ M Oligo d(T)16 (Roche, Mannheim, Germany) and 2.5 IU/ μ l MML-V RTase (Promega, Tokyo, Japan) in a total volume of 20 μ l. cDNA proceeded with the following PCR cycling program: 94°C denaturation for 5 minutes, then 33 cycles of 94°C for 30 seconds, 59°C for 30 seconds, 72°C for 30 seconds, and an extra extension for 5 minutes at 72°C. The following primers were used in this study: human *klotho* (forward) 5'-ACTCCCCCAGTCAGGTGGCGGTA-3', (reverse) 5'-TGGGCCCGGGAACCATTCG TGTC-3'⁵²; human glyceraldehyde 3-phosphate dehydrogenase (forward) 5'-GAAGGTGAAGGTCTG GAGT-3', (reverse) 5'-GAAGATGGTGATGGGATTTC-3'. PCR products were seen on a 2% agarose gel.

Western blot analysis

Total protein was extracted with protein prep kit (Qiagen, Hilden, Germany) and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). The blotted membranes were incubated overnight with primary antibodies against klotho (Abcam, Cambridge, MA, USA) and β -actin (Sigma-Aldrich Inc.) at 4°C. Membranes were then incubated 1 hour with horseradish-peroxidase-conjugated secondary antibodies at room temperature. Protein expression was detected with the ECL system (Santa Cruz Biotechnology).

Immunohistochemical staining

Hair follicles were fixed and embedded in paraffin. Staining was performed on 5 μ m paraffin sections. After deparaffinization and rehydration, sections were incubated with a rabbit monoclonal antibody specific for human klotho (1:200; Abcam) for 3 hours at room temperature. Antibody binding was checked by UltraVision LP detection system (Thermo Fisher Scientific Walt, Waltham, MA, USA) according to the manufacturer's instructions. 3-3' diaminobenzidine (Thermo Fisher Scientific Walt)

was used to color development.

Immunofluorescence staining

For Ki-67 staining, sections were incubated with mouse monoclonal Ki-67 antibody (Abcam) and incubated with Alexa Flour 488 labeled donkey anti-mouse secondary antibody (Abcam). Specimens were measured using a Leica TCS-SPE confocal microscope (Leica Microsystems, Bannockburn, IL, USA).

For terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, sections were treated with 20 μ g/ml of proteinase K (Millipore Corp., Billerica, MA, USA) for 15 minutes at room temperature and incubated with terminal deoxynucleotidyl transferase enzyme (Millipore corp.) for 60 minutes at room temperature. TUNEL-positive cells were detected by an anti-digoxigenin fluorescein antibody. Specimens were then stained with propidium iodide (Sigma-Aldrich Inc.) and seen using a Leica TCS-SPE confocal microscope (Leica Microsystems). The number of TUNEL positive cells were counted and analyzed.

Statistical analysis

Data management were analyzed by the IBM SPSS Statis-

tics ver. 20.0 (IBM Corp., Armonk, NY, USA) for Windows statistical program. Statistical analysis was done with one-way ANOVA, then by Dunnett's multiple comparison tests to compare three or more groups. The Student's t-test was employed to compare each group. A *p*-value less than 0.05 was considered to be statistically meaningful. All data are

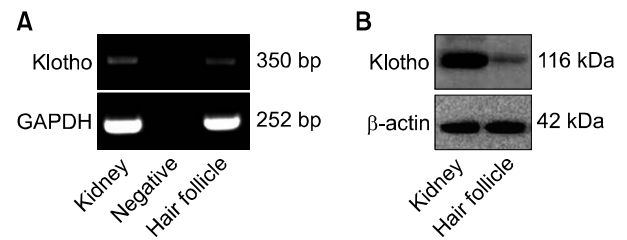


Fig. 1. Expression of *klotho* mRNA and protein in human hair follicles. (A) Reverse transcription polymerase chain reaction was performed on isolated anagen VI stage hair follicles using a specific *klotho* primer pair. Human kidney tissue was used as a positive control and PBS (no cDNA) was used as a negative control. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. (B) Western blot analysis was performed on isolated anagen VI stage hair follicles using a specific *klotho* antibody. Human kidney tissue was used as a positive control. β -actin was used as a loading control.

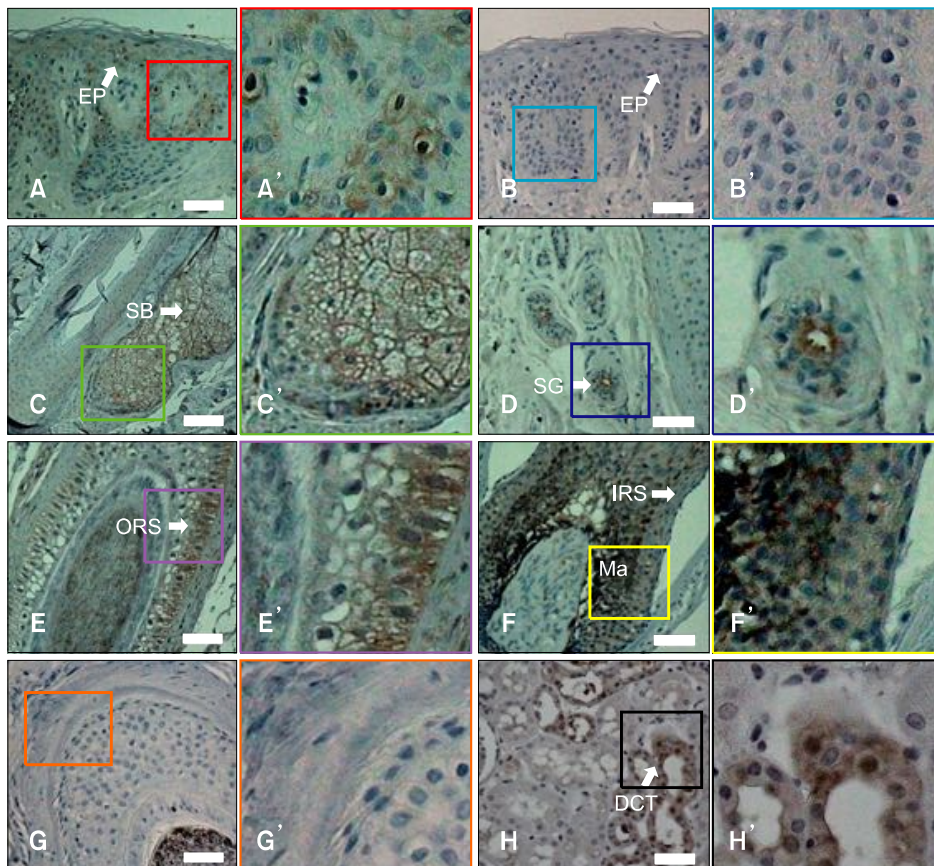


Fig. 2. Immunohistochemical localization of *klotho* in human scalp skin. Paraffin sections of human scalp skin (A~G) and human kidney tissue (H) were stained with anti-*klotho* antibody. Close-up images of boxed regions of A~H were also shown in A'~H'. Negative control was performed with normal rabbit immunoglobulin G instead of primary antibody in the sections of human hair follicle units (B and B'; G and G'). Positive control was performed with anti-*klotho* antibody in the sections of human kidney tissue (H and H'). EP: epidermis, SB: sebaceous gland, SG: sweat gland, ORS: outer root sheath, IRS: inner root sheath, Ma: hair matrix, DCT: distal convoluted tubules. Scale bar = 100 μ m.

presented as the mean \pm standard deviation of at least three separate experiments.

RESULTS

Klotho expresses in human hair follicles

The expression of *klotho* mRNA in hair follicles was identified by RT-PCR analysis with a specific *klotho* primer pair. A DNA band corresponding to 350 bp was confirmed in human hair follicles (Fig. 1A). The human kidney tissue served as the positive control⁷, produced a band similar to that in hair follicles (Fig. 1A). Presence of the klotho protein in human hair follicles was identified by western blot analysis with a specific klotho antibody. Klotho protein was detectable in human hair follicles with the expected molecular weight of 116 kDa. Human kidney tissue as the positive control, produced a band similar to that in the hair follicles (Fig. 1B).

Klotho immunoreactivity is detected in the epithelium of human skin and hair follicles

Immunohistochemical staining was performed on human scalp skin using a polyclonal rabbit antibody against human klotho. Klotho immunoreactivity was detected in both anagen hair follicles (Fig. 2E, E'; F, F') and extra-follicular structures (Fig. 2A, A'; C, C'; D, D'), while

staining was not detected in negative controls (Fig. 2B, B'; G, G'). In human skin, klotho immunoreactivity was mainly expressed in the epithelial cells (Fig. 2A, A'). In human hair follicles, specific immunoreactivity for klotho was found in the outermost areas of outer root sheath (ORS; Fig. 2E, E'). Moderate immunoreactivity for klotho was found in the hair matrix and region of the inner root sheath (Fig. 2F, F'). Klotho positive cells were also found in the sebaceous glands (Fig. 2C, C') and sweat glands (Fig. 2D, D'). Human kidney tissue, as a positive control, was also expressed klotho in the region of distal convoluted tubules (Fig. 2H, H').

Klotho expression decreases with aging

To determine the aging dependent expression pattern of klotho in human hair follicles, individual hair follicles (a small piece of scalp skin, aged from 21 to 70) were collected, and klotho expression was identified by western blot assay and immunohistochemistry. Klotho expression was decreased with aging (Fig. 3A) and klotho immunoreactivity was mainly reduced in the epithelial cells of the epidermis and ORS region (Fig. 3B).

Klotho silencing results in hair growth inhibition and premature catagen induction

After transfection with *klotho* siRNA, hair follicles were

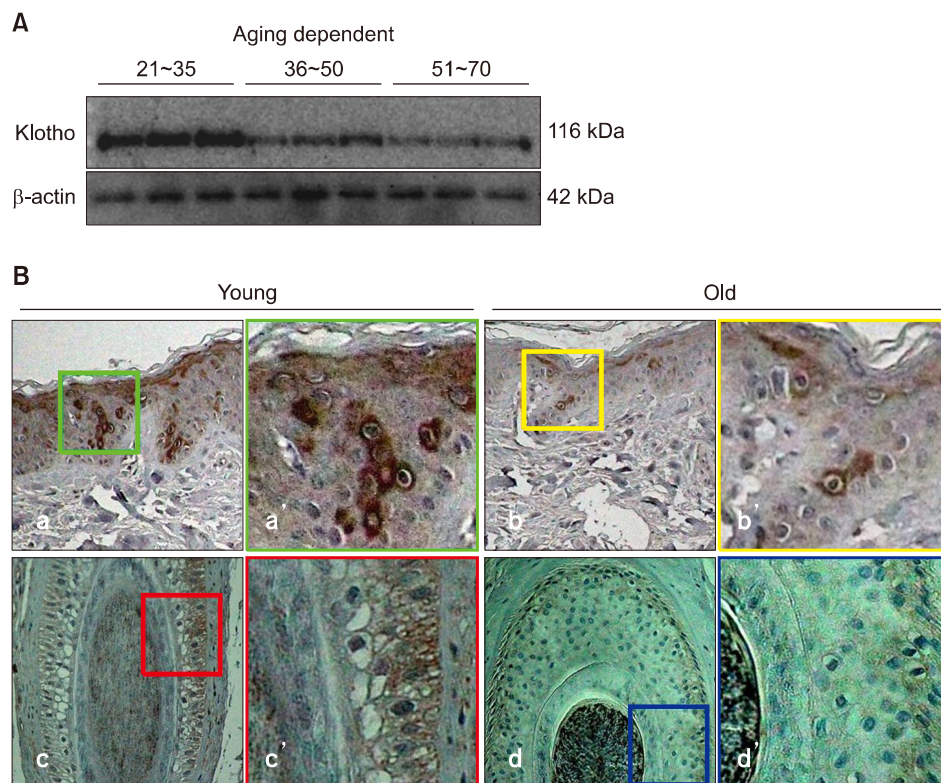


Fig. 3. Aging dependent expression pattern of klotho in hair follicles. (A) Western blot analysis was performed on individual human hair follicles (aged from 21 to 70). β -actin was used as a loading control. (B) Paraffin sections of young (28 years) and old (65 years) human hair follicle were stained with anti-klotho antibody (a~d). Close up images of boxed regions of a~d were also shown in a'~d'. a and a', Klotho immunoreactivity in the epidermis from young subject. b and b', Klotho immunoreactivity in the epidermis from aged subject. c and c', Klotho immunoreactivity in the outer root sheath from young subject. d and d', Klotho immunoreactivity in the outer root sheath from aged subject. Scale bar=100 μ m.

cultured over three days. Hair follicle length and hair cycle score were measured. *Klotho* siRNA treated hair follicles ($1,058.58 \pm 166.82 \mu\text{m}$) had significantly reduced hair growth compared with non-targeted control siRNA treated hair follicles ($692.35 \pm 99.35 \mu\text{m}$) on the third day (Fig. 4A).

Quantitative morphometry of the hair follicles was revealed that *klotho* silencing accelerates spontaneous catagen development (Fig. 4B). It was confirmed by calculation of the hair cycle score (Fig. 4C), which was significantly ($p < 0.05$) increased in the *klotho* siRNA treated hair follicles (233.74 ± 10.15) compared with the non-targeted control siRNA treated hair follicles (121.27 ± 9.93), indicates that *klotho* silencing results in premature catagen induction.

Quantitative histomorphometry of TUNEL was revealed that TUNEL positive cells were significantly ($p < 0.05$) increased in *klotho* siRNA treated hair follicles (43.75 ± 5.93) compared with the controls (23.33 ± 4.16) (Fig. 4D,

E). However, immunoreactivity of the cell proliferation marker Ki-67 was not significantly changed between *klotho* siRNA treated hair follicles and the control hair follicles (Fig. 4F, G).

Klotho protein prolongs hair growth and anagen phase

Next, we examined whether treatment with klotho protein resulted in any change in hair growth. Hair follicles were treated with $0.1 \mu\text{g/ml}$ or $0.5 \mu\text{g/ml}$ klotho protein every other day and cultured for 8 days. Treatment with klotho significantly ($p < 0.05$) increased hair follicle length ($1,764.59 \pm 150.59 \mu\text{m}$) compared with the control hair follicles ($1,427.42 \pm 137.78 \mu\text{m}$) (Fig. 5A).

Quantitative morphometry of the hair follicles was revealed that klotho treatment delays catagen development (Fig. 5B). Hair cycle score was significantly ($p < 0.05$) reduced in the protein-treated hair follicles (171.48 ± 16.15) compared with control hair follicles (248.32 ± 18.93) (Fig. 5C).

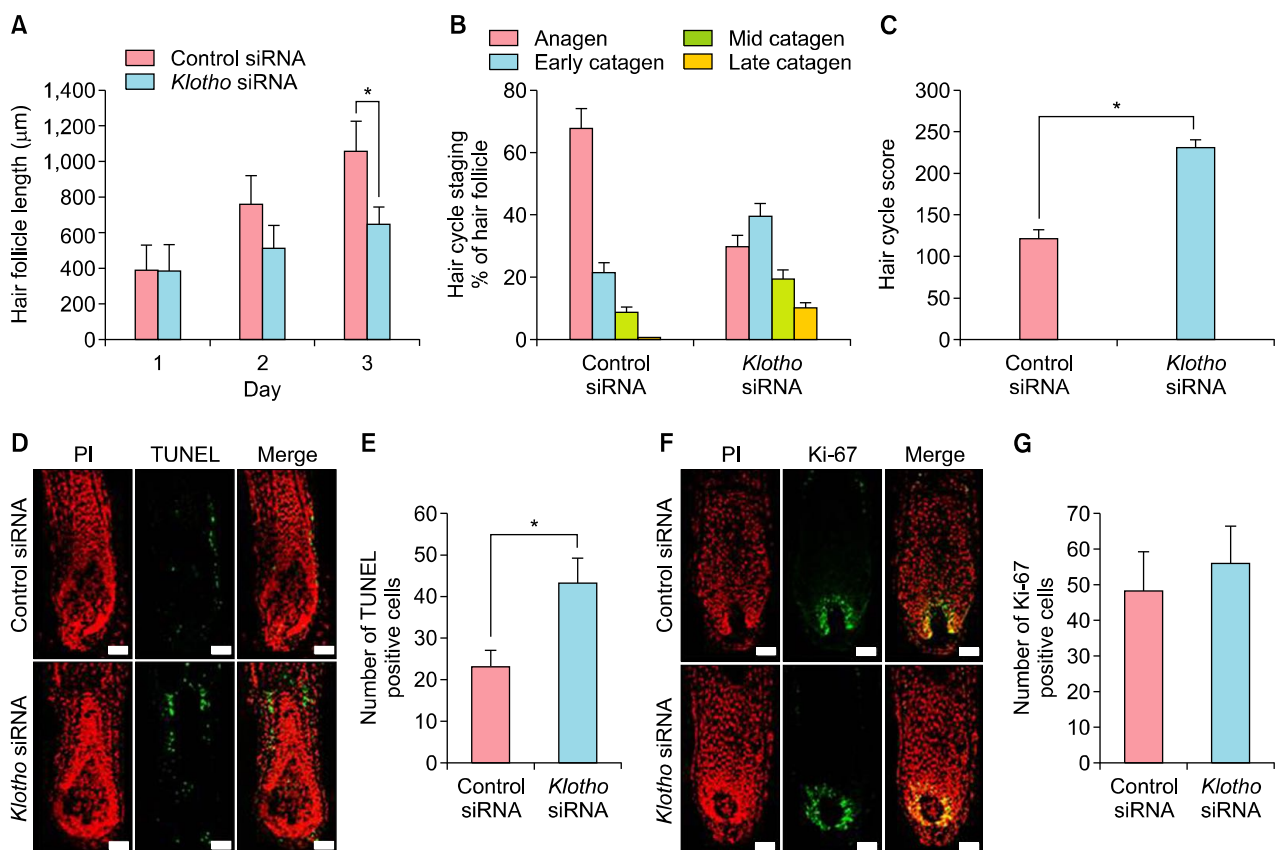


Fig. 4. Effects of *klotho* silencing on human hair growth and hair cycling. Hair follicles were transfected with *klotho* siRNA (200 nM) or non-targeting control siRNA. (A) The length of hair follicle unit was measured every day until 3 days. (B) Measurement of hair cycle staging of each hair follicle (anagen, early catagen, mid catagen, late catagen). (C) Calculation of hair cycle score of each hair follicle. (D) Representative images of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive cells (green) in control or *klotho* siRNA treated hair follicles. (E) Quantification of cell apoptosis. (F) Representative images of Ki-67 positive cells (green) control or *klotho* siRNA treated hair follicles. (G) Quantification of proliferative cell. Data are based on 40~50 hair follicle units per group, and are presented as the mean \pm standard deviation. * $p < 0.05$. Scale bar = $100 \mu\text{m}$. PI: propidium iodide.

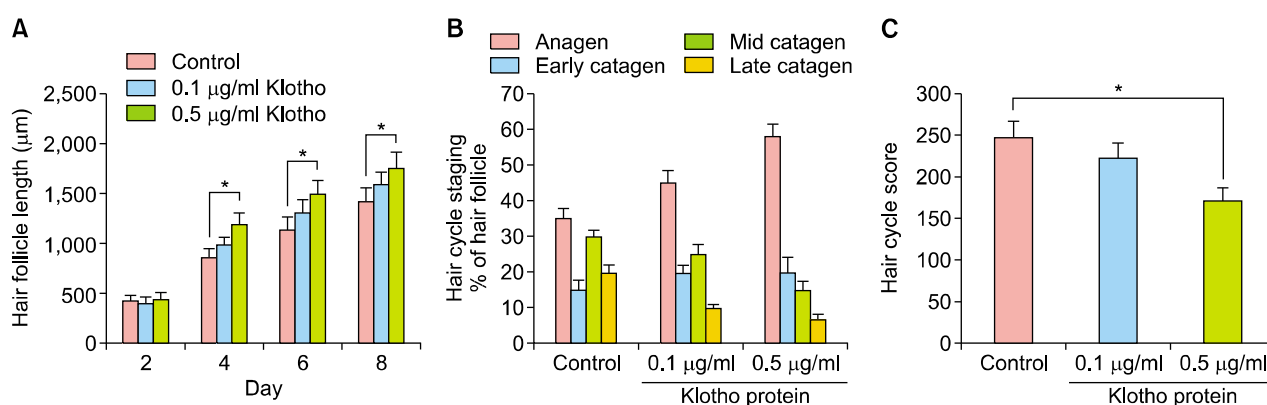


Fig. 5. Effects of klotho protein on human hair growth and cycling. Hair follicles were treated with klotho protein (0.1 or 0.5 $\mu\text{g/ml}$) every other day. Hair follicle organ culture was performed over 8 days. (A) The length of hair follicle unit was measured every other day until 8 days. (B) Measurement of hair cycle staging of each hair follicle (anagen, early catagen, mid catagen, late catagen). (C) Calculation of hair cycle score of each hair follicle. Data are based on 40~50 hair follicle units per group, and are presented as the mean \pm standard deviation. * $p < 0.05$.

DISCUSSION

Klotho has been identified as an “aging-suppressor” gene in mammals that expedites aging upon disruption and boosts that life expectancy upon over-expression^{9,17}. To date, the expression of *klotho* has only been partly deciphered. *Klotho* is expressed in the kidney, brain, prostate, placenta, pancreas, skeletal muscle, testis, ovary, inner ear and small intestine^{10,13,18}. However, it is uncertain whether *klotho* is presented in human hair follicles. We observed that *klotho* is expressed in human hair follicles at both gene and protein levels (Fig. 1). In hair follicles, *klotho* immunoreactivity was mainly observed in the outermost areas of the ORS and hair bulb matrix cells (Fig. 2). Multiple aging-like phenotypes have been first documented in *klotho*-deficient mice. Skin atrophy and hair loss are also present in this model⁶. To date, whether *klotho* expression is correlated with human hair aging has not been clarified. Our results showed that *klotho* expression is decreased with aging (Fig. 3A) and *klotho* immunoreactivity is mainly reduced in the epithelial cells of epidermis and ORS region (Fig. 3B). Hair loss and graying are common symptoms of aging^{19,20}. Androgenetic alopecia is the most common form of hair loss, which shows clear age-related progression²¹. Furthermore, some authors have put forward a unique age-related entity, referred to as senescent alopecia^{21,22}. Taken together, these results show that *klotho* may be associated in the regulation of hair aging.

To clarify the functional role of *klotho* in hair growth and cycling, anagen hair follicles were transfected with human *klotho* siRNA as described previously^{16,23}. In situ knock-down of *klotho* was observed at the gene and protein levels in organ-cultured hair follicles. Furthermore, we ob-

served that *Klotho* silencing lead to the premature catagen induction and hair growth inhibition (Fig. 4). We also confirmed that *klotho* silencing promotes cell apoptosis in follicular keratinocyte (Fig. 4). Otherwise, recombinant *klotho* protein treatment increases hair follicle length and delays catagen development (Fig. 5). During hair growth phase, multiple factors promoting proliferation, differentiation and survival predominate, whereas hair regression is characterized by activation of various signaling pathways that induce apoptosis in the hair matrix, inner root sheath and ORS keratinocytes^{24,25}. Our data suggest that *klotho* might be involved in the hair cycle through inhibition of cell apoptosis and might operate as a major molecular brake on hair follicle regression.

In conclusion, *klotho* was expressed in human hair follicles at both gene and protein levels. In hair follicles, *klotho* expression was prominently observed in the outermost area of ORS and hair bulb matrix cells. Quantification of *klotho* expression in young and aged donors revealed that *klotho* immunoreactivity decreased with aging. In hair follicle organ culture, *klotho* silencing induced premature catagen entering and inhibited hair growth. Otherwise, *klotho* protein prolonged human hair growth. These results indicate that *klotho* might be an important regulatory factor for human hair growth and hair cycle change.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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