Morphological characteristics and factors in the plucked human hair follicle tissue of curved hair caused by acquired factors

Horibe, Ippei[1*]; Ke, Yuru[2]; Izumi, Sara[2]; Ishihara, Ryoji[1]; Nakano, Takaya[1]; Sumiyoshi, Takaaki[2]; Nagaoka, Yasuo[2]

[1] Research & Development Division, NAKANO SEIYAKU Co., Ltd., Kyoto, Japan;
[2] Department of Life Science and Biotechnology, Kansai University, Suita, Osaka, Japan

***** Horibe, Ippei; 6-20 Kitainouecho, Higashino, Yamashina-ku, Kyoto, 607-8141 Japan;
+81-75-502-7310; ippei_horibe@nakano-seiyaku.co.jp

Abstract

Background: In addition to congenital curly hair, "curved hair" caused by acquired factors is thought to become worse the impression of human physical appearance, however the detailed mechanism is still obscure. We previously demonstrated that hair follicle tissue cross section of curved hair was asymmetrical compared to identical participant's straight hair. Here, we attempt to clarify the causes of curved hair which seems to have occurred by acquired factors.

Methods: The straight and curved hair with hair follicle tissue attached were collected from the identical participants and the differences were evaluated by morphological and immunohistochemical analysis of hair follicle tissue cross section.

Results: We found that the plucked hair follicle tissue had distorted shapes, in curved hair obtained from participants in their 30s to 50s. Furthermore, it was observed that hair matrix cells based on multiple hair germs fused to eventually form a single hair follicle and hair shaft. In the curved hair follicles, KRT71, a type of keratin protein involved in the differentiation of inner root sheath, was unevenly present and the protein expression level of DKK1, an inhibitor of the Wnt signaling pathway, was elevated.

Conclusion: Our study revealed the hair matrix cell fusion in the middle of hair cycle was one of the causes of acquired curved hair. We consider that the fusion of independent hair matrix cells with different vertical positions causes partial differences in the timing of differentiation, resulting in incomplete (asymmetric) hair follicle shapes and hair curving.

Keywords: curved hair; hair matrix cell fusion; hair follicle morphogenesis; KRT71; Wnt signaling pathway

Introduction.

The appearance of hair changes depending on many factors such as shape, color, and optical properties, and greatly affects the first impression of the human physical appearance. Hair shows various forms such as straight hair and curled hair depending on individual and racial differences [1], and its form is strongly influenced by the shape of hair follicle. That is, it is considered that the hair follicle of "straight hair" is straight, while the hair follicle of "curly hair" are bent [2]. In addition to congenital factors, acquired factors may cause hair curving, and in fact, there is report that hair curving occurs with aging [3]. The increase in acquired curly hair i.e., "curved hair" is considered to become worse the impression of appearance because it leads to the disordered alignment of hair fibers, resulting in a decrease in hair luster.

The curling of wool is caused by the presence of two types of cortex cells, orthocortex cells and paracortex cells, on the outside and inside of wool, respectively [4]. Similarly, there are reports that the distribution of two kind of cortex cells affects hair shape in human hair [5,6]. On the other hand, regarding the difference between the hair follicles of straight hair and curly hair, Thibaut et al. reported that in follicles of curled hair such as Africans, hair follicle tissue becomes asymmetrical, resulting in hair curling because of mechanical action [7]. Furthermore, as for the difference in the expression of proteins in hair follicles, there are reports that mRNA of hHa8, a type of keratin protein, is highly expressed in concave side (inside the bent) of curled hair follicle tissue [8]. There is also a report that Insulin like growth factor binding protein 5 (IGFBP5), a related protein of cell growth factor IGF, is over-expressed in the convex side (outside the bent) [9]. However, there are few reports based on embryological perspectives on hair curving caused by aging.

The structure of the entire hair follicle repeating the hair cycle is divided into a permanent portion which includes the sebaceous gland and the bulge region, and a temporary portion that is underlying the bulge region [10]. That is, unlike many other organs, it is expected that the hair follicle morphogenesis may be susceptible to aging, since the developmental process is repeated many times according to the hair cycle. In this study, we hypothesized that some

adverse effects with aging prevent the formation of normal hair follicle tissue and result in acquired curved hair. Therefore, by using plucked human hair follicle tissues of straight hair and curved hair obtained from the identical participant's in their 30s-50s, we examined their differences in morphological characteristics. Hair matrix cells based on different hair germs fused in hair follicle to eventually form a single hair shaft. We found that multiple hair matrix cells may fuse to form a single hair, resulting in acquired curved hair. Therefore, we considered that failure occurred in Wnt signaling pathway due to some factors such as aging, and multiple hair matrix cells fused to form uneven hair follicle tissue, contributing to the formation of acquired curved hair. We believe that these results are novel discoveries that will help to improve acquired curved hair.

Materials and Methods.

Specimen collections

Hairs were plucked from the participant's scalp, and the tissues of the attached follicle were used for histological evaluation. The participants were men and women in their 30s-50s, and straight hair and curved hair were collected from the identical participant, respectively. All experimental procedures using human hair samples were conducted according to the Declaration of Helsinki Principles, and written informed consents were obtained from all participants.

Scanning electron microscopy (SEM) analysis

The sample was prepared according to the conventional SEM analysis method. To be brief, a dry sample was set on a brass sample table and gold coating was performed using an ion sputtering apparatus JFC 1100 (JEOL, Tokyo, Japan). The image of the hair sample was taken at 30 kV and magnification 500 times using a scanning electron microscope JSM 6390LV (JEOL).

Preparation and staining of thin paraffin sections

Hair follicle tissues, obtained by the above-mentioned pluck method, were fixed in 4% phosphate-buffered paraformaldehyde overnight at 4°C and embedded in paraffin. Continuous cross sections with a thickness of 10μm were obtained using a rotary microtome

RX-860 (Yamato Kohki, Saitama, Japan) and mounted onto MAS-GP Adhesive Microscope Slide glass (Matsunami, Osaka, Japan). After the thin sections were thoroughly dried, to remove paraffin, slides were washed for 5 minutes in xylene, 100% EtOH, 90% EtOH, and 80% EtOH, followed by 3-minutes rehydration in dH_2O. Hematoxylin and eosin (H&E) staining was performed using Meyer's hematoxylin solution (Merck, Kenilworth, NJ, USA) and eosin Y solution (Nacalai Tesque, Kyoto, Japan).

Immunohistochemical staining

The deparaffinated slides were immersed in a HistoVT One (Nacalai tesque) solution heated to 90°C in hot water bath, and they were heated for 20 minutes to reactivation the antigen. After antigen retrieval, slides were rinsed 3 times in PBS for 5 minutes each. To quench the endogenous oxidase, the slides were treated with 10% H_2O_2 in PBS for 30 minutes. Finally, primary antibodies, secondary antibodies, streptavidin-horseradish peroxidase and 3,3'-diaminobenzidine tetrahydrochloride (DAB: Dojindo, Kumamoto, Japan) were applied to detect the protein of interest. Primary antibodies were one of the following: Trichohyalin mouse monoclonal antibody (Abcam, Cambridge, United Kingdom), KRT71 rabbit polyclonal antibody, CUTC rabbit polyclonal antibody, TGF-β1 rabbit polyclonal antibody, TGF-βRI rabbit polyclonal antibody (GeneTex, San Antonio, TX, USA), Wnt5a rabbit polyclonal antibody, Wnt3a rabbit polyclonal antibody (Bioss, Woburn, MA, USA) , Frizzled6 rabbit monoclonal antibody (Cell Signaling, Danvers, MA, USA) and DKK1 rabbit polyclonal antibody (Proteintech, Chicago, IL, USA). The secondary antibodies used horseradish peroxidase-labeled anti-mouse and anti-rabbit antibody (SeraCare, Milford, MA, USA).

Results.

Observation of characteristic forms found in acquired curved hair

Straight hair and curved hair with hair follicle tissue attached were obtained from some participant's in their 30s - 50s having hair thought to have curved by acquired factors. The obtained plucked hair follicle tissues were embedded in paraffin, and continuous cross sections were obtained from the root side with a thickness of 10μm. Thereafter, paraffin

sections were stained with Hematoxylin staining, and the morphological differences between straight hair and curved hair were observed (Figure-1.).

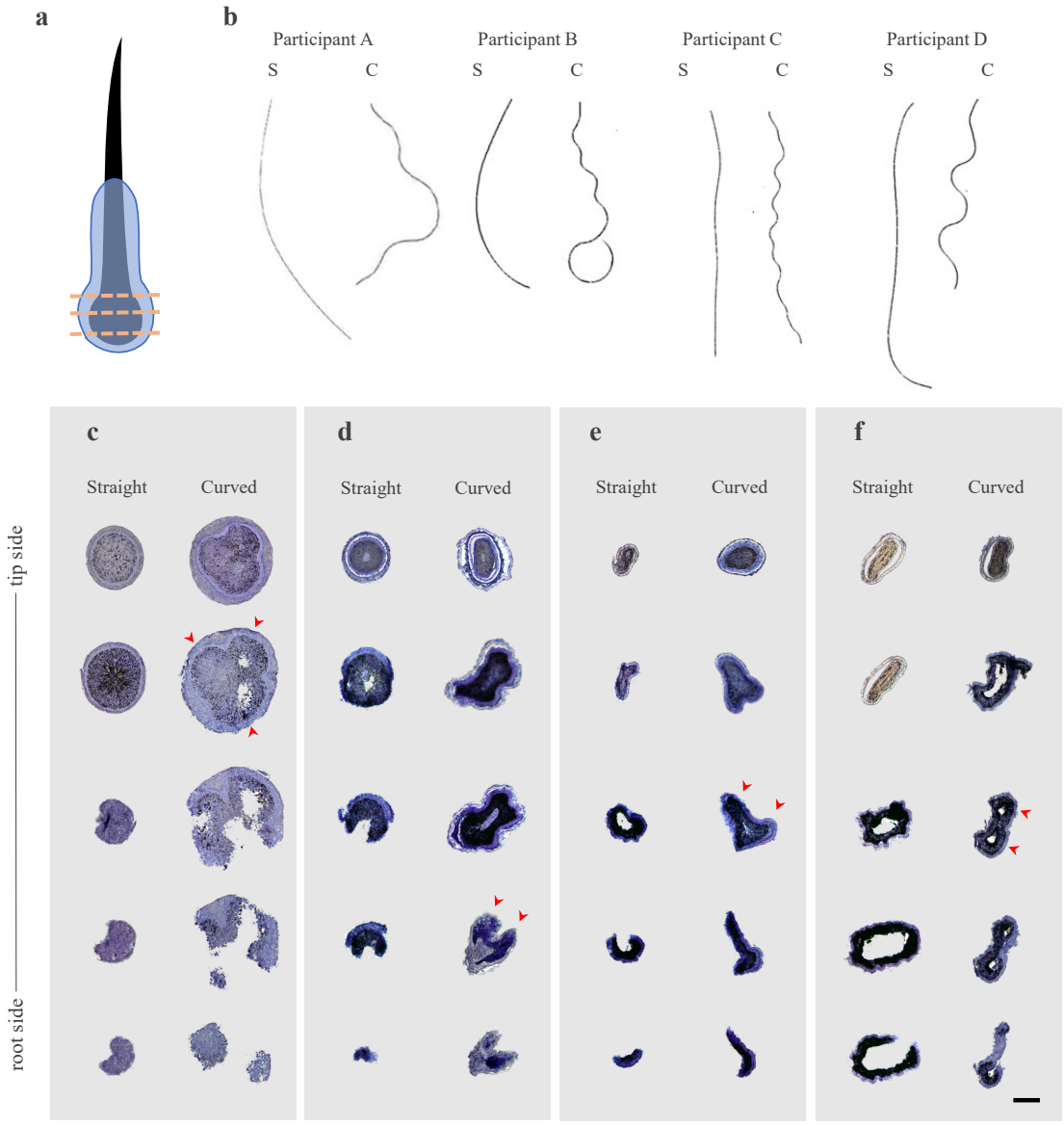

Figure-1. Observation of plucked hair follicle tissue cross sections. Continuous cross sections were obtained using plucked hair follicle tissue of straight hair and curved hair obtained from multiple participants. Histological observations were performed by brightfield imaging by hematoxylin staining. **a**: Schematic diagram of hair follicle tissue, the dashed line is the cutting direction, **b**: Shape of hair shaft of each sample, S: Straight hair, C: Curved hair, **c-f**: Typical observation results of "participant A-D", respectively, the red arrowhead indicates the part where the hair matrix cell fusion, Scale bar: 100μm

As a result, although it may be affected by removal, it was found that the hair follicle tissue is formed in a shape close to the perfect circle in the straight hair, while in the curved hair, the hair was not a round circle, and the inner root sheath is also distorted. In the curved hair of all participants, it was observed that multiple hair matrix cells fused to form a single hair. In the "participant A" curved hair, where the hair matrix fusion was clearly observed, three hair matrix cells thought to have been originally independent fused, and inner root sheath was formed to cover them, eventually forming a single hair follicle (Figure-1.). On the other hand, it was observed that single hair follicle was formed from single hair matrix cell, in the straight hair. In the straight hair, single hair matrix cell formed single hair follicle, however in participant C and D, a slightly distorted shape was observed in the hair follicle tissue of their straight hair (Figure-1.c, d). Tissue shedding was observed near the center of the hair matrix cell, but this part is considered to be the part where the dermal papilla cells dropped out when the hair was plucked. In addition, when the hair shaft of "participant A" was observed with a scanning electron microscope, it was found that the curved hair was thicker in diameter than the straight hair and had a partially bulging shape (Figure-2).

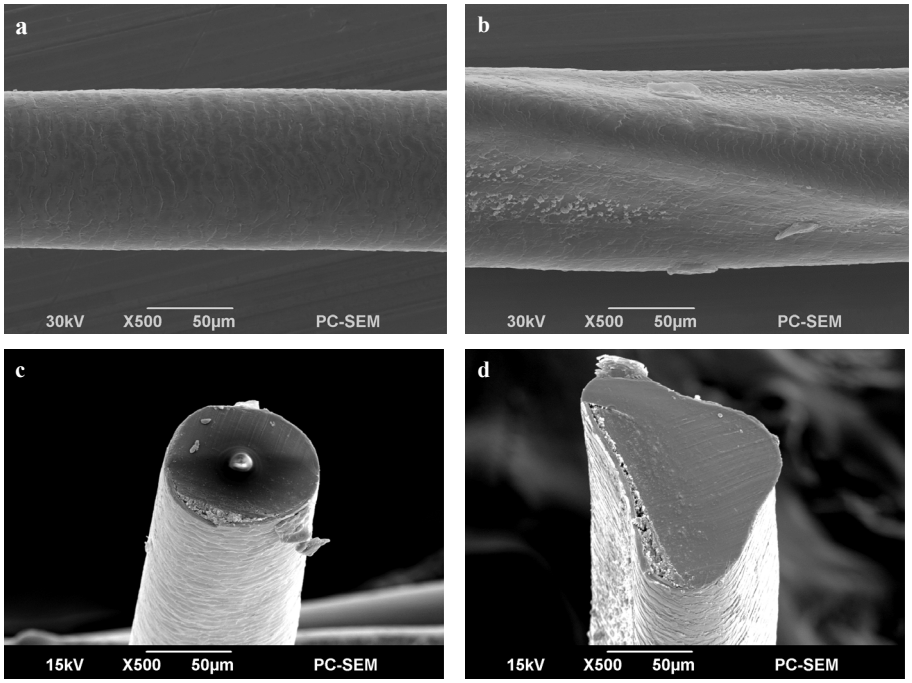

Figure-2. SEM observation image of straight and curved hair in "participant A".

SEM observation image of the plucked hair, **a, c**: straight hair, **b, d**: curved hair

Immunohistochemical staining of plucked hair follicle tissue

In order to investigate the cause of the difference in the morphological characteristics of acquired curved hair, immunohistochemical staining was performed on factors thought to be involved in the morphogenesis of hair follicles using the plucked hair follicle tissue of "participant A". As a result, it was found that protein expression levels of KRT71 [11], a type of keratin protein specific to inner root sheaths, and CUTC [12], a factor involved in copper transport, had uneven in the hair follicle tissue of curved hair (Figure-3.).

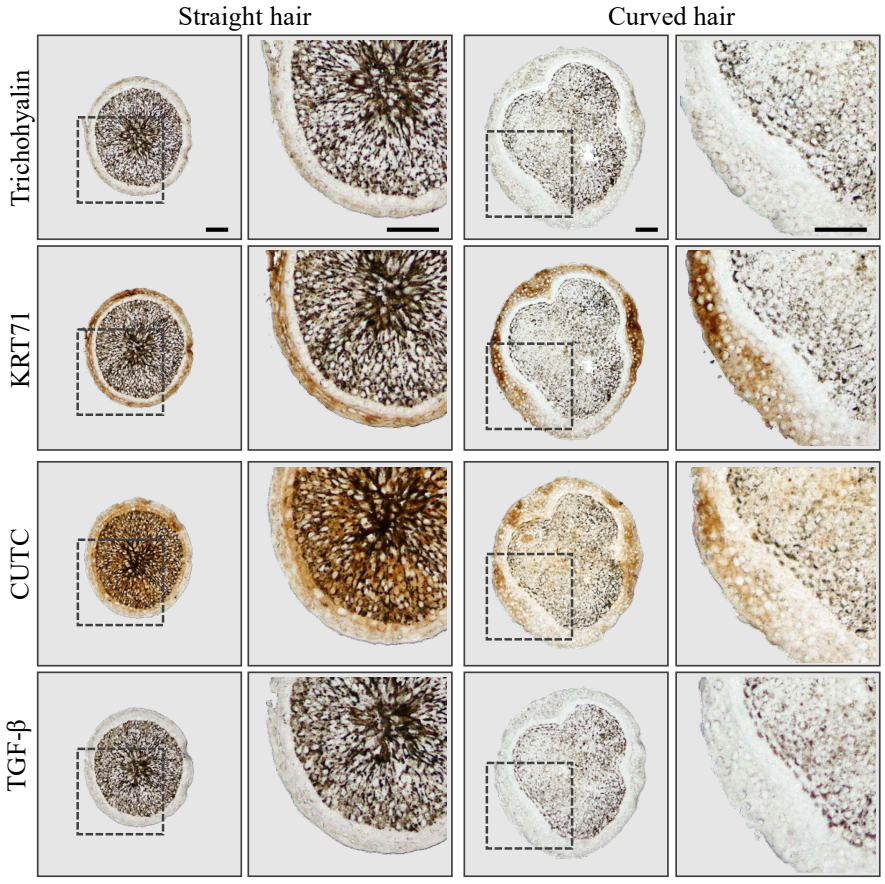

Figure-3. Immunohistochemical staining of plucked hair follicle tissue in "participant A". Immunohistochemical analysis of plucked hair follicle tissue was performed by DAB staining using anti-trichohyalin antibodies, anti-KRT71 antibodies, anti-CUTC antibodies and anti-TGF-β antibodies. The straight hair was shown in the left two columns and the curved hair was shown in the right two columns. The enlarged views of the black dashed line area are shown at the right, respectively. Scale bar: 30μm

Study on factors involved in induction of differentiation of hair follicle tissue

We thought that the multiple hair matrix fusion in the curved hair follicle tissue may have caused a difference in the timing of partial differentiation, and we investigated the factors of the Wnt signaling pathway involved in the developmental process. As a result, there was no difference in protein expression levels of Wnt3a, which is involved in the Wnt-β catenin pathway responsible for cell proliferation, differentiation, and organ formation, and Frizzled6, a receptor for Wnt. On the other hand, in the curved hair, the protein expression level of Wnt5a, which is involved in the planar cell polarity pathway (Wnt-PCP pathway), which regulates the cell polarity of the developmental process, partially reduced. In addition, in the curved hair, the protein expression level of DKK1, an inhibitor of the Wnt-β catenin pathway, was elevated (Figure-4.).

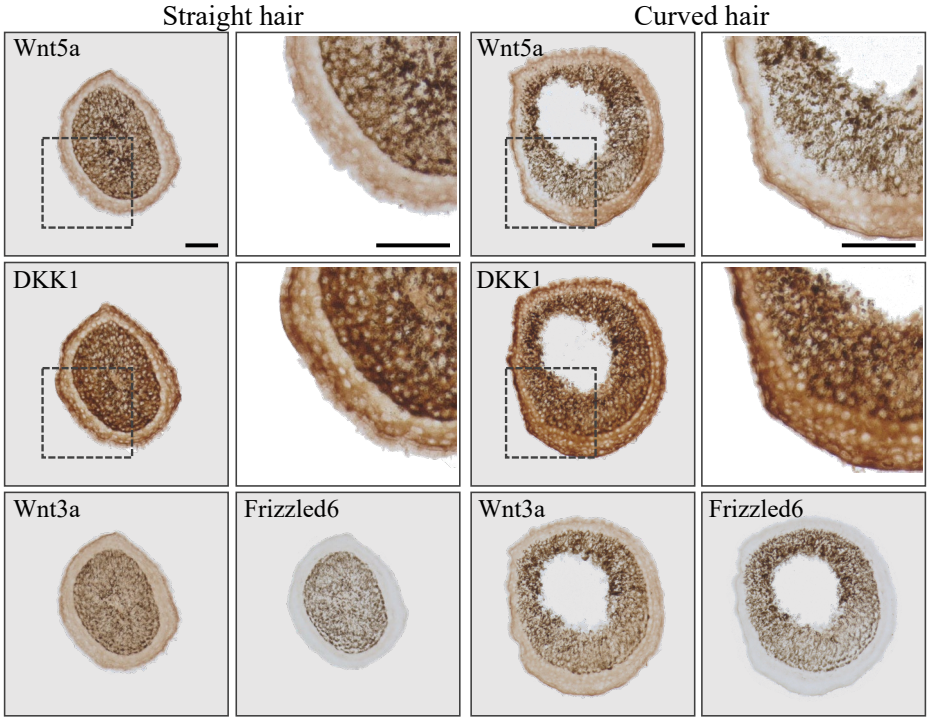

Figure-4. Comparison of Wnt-related factor expression in "participant E". Immunohistochemical analysis of plucked hair follicle tissue was performed by DAB staining using anti-Wnt5a antibodies, anti-DKK1 antibodies, anti-Wnt3a antibodies and anti-Frizzled6 antibodies. The straight hair was shown in the left two columns and the curved hair was shown in the right two columns. The enlarged views of the black dashed line area are shown at the right, respectively. Scale bar: 100μm

Discussion.

In this study, based on the hypothesis that some adverse effects with aging prevent the formation of normal hair follicle tissue and result in acquired curved hair, we examined the difference in morphological characteristics using plucked hair follicle tissues of straight hair and curved hair obtained from the identical participants in their 30s-50s. From the observation of the cross section of the plucked hair follicle tissues obtained from the identical participant, it was observed that hair matrix cells based on different hair germs fuse with each other in a curved hair follicle, eventually forming a single hair shaft (Figure-1.). The observation results with a scanning electron microscope also presumed the possibility of multiple hairs fusion (Figure-2.). As for reports of multiple hairs in single hair follicle, there is alopecia with scarring common in African women called Central centrifugal cicatricial alopecia (CCCA) [13,14]. In cases called Pili multigemini, which are occasionally found in children's hair and beards, it has been reported that multiple hair shafts are formed in single hair follicle or that they have a hair shaft partially integrated into one [15]. As for the fusion of hair, the above-mentioned cases have been reported, however it is a relatively rare case, and there is no mention of viewpoints such as causes of acquired curved hair.

As a result of immunohistochemical staining, it was found that the expression of KRT71 [11], a type of keratin protein involved in the differentiation of the inner root sheath, was uneven in hair follicle tissue of curved hair (Figure 3). That is, it is shown that there is a partial difference in the differentiation stage in the inner root sheath tissue of the curved hair. Therefore, when factors related to the Wnt signaling pathway, which is important in organ development, were investigated, Wnt5a partially reduced, and DKK1 increased in curved hair (Figure-4.). Recently, Iwasaki et al. reported that inhibiting the planar cell polarity pathway (Wnt-PCP pathway), which regulates the cell polarity of the developmental process, causes abnormalities in the direction in which the scales extend in zebrafish [20]. Although we lack definite information about the causes of hair matrix fusion, we suspect that abnormalities in the Wnt signaling pathway may be involved in that.

In the normal scalp, hair organ is consisted of one to four hair follicles, forming an anatomical unit called a hair follicular unit along with the sebaceous glands and arrector pili muscle [17]. In the hair follicular unit, each hair is reborn with an independent hair cycle,

and it is considered that the distance between each hair papillae and secondary germ keep at a certain distance. Considering the result of our study, it was suggested that some factors prevent normal hair follicle regeneration from being maintained, which can lead to closer the distance between the secondary hair germs in the hair follicular unit, result in the fusion of hair matrix cells. We consider that the fusion of hair matrix cells with different positions in the depth and the timing of differentiation forms hair follicles with partial differences in the thickness of the follicle sheath. As a result, the shape of the hair becomes distorted (asymmetric), and curved hair is generated (Figure-5.).

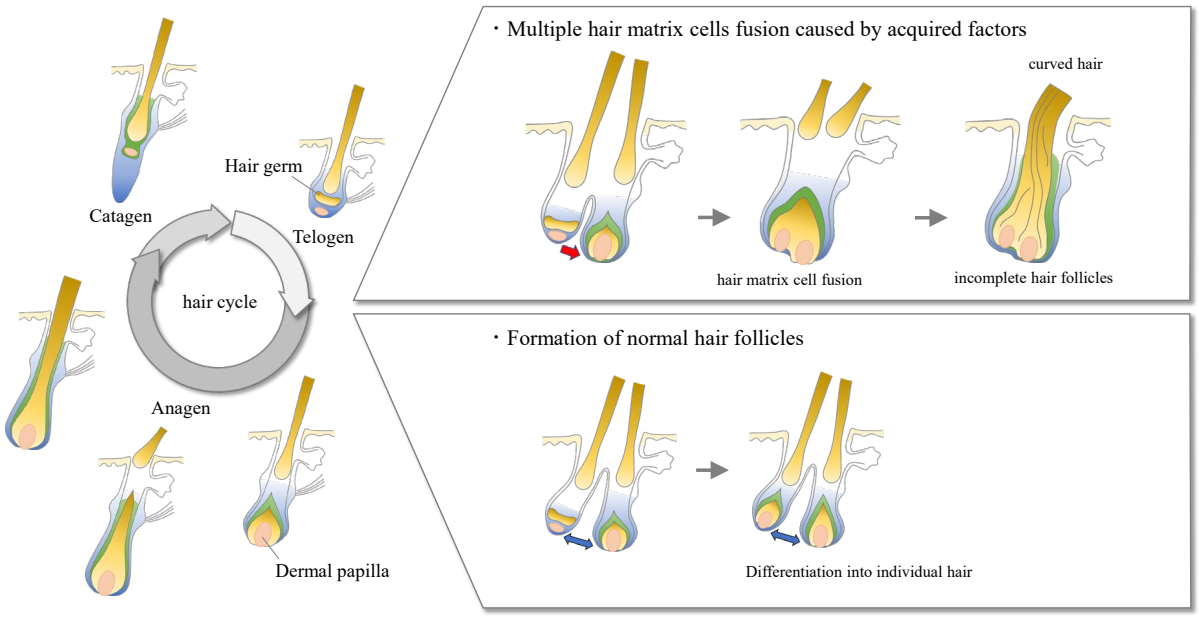

Figure-5. Schematic diagram of hair cycle and the expected formation process of curved hair caused by acquired factors. Hair is reborn in a 4-6 years cycle through a series of processes called "Anagen", "Catagen", and "Telogen", and this is called the hair cycle. In the hair cycle, a temporary portion of the hair follicle that is underlying the bulge region is reduced with the Catagen phase and reshaped with a new Anagen phase. Usually, hair organ is consisted of one to four hair follicles at a constant distance, forming an anatomical unit called a hair follicular unit along with the sebaceous glands and arrector pili muscle. If hair follicles affected in some acquired factors during the new Anagen phase, we considered that the secondary hair germs in the hair follicular unit cannot keep the distance constant, and result in the fusion of hair matrix cells.

Conclusion.

In order to examine the curved hair caused by acquired factors, the difference was observed by comparing the plucked hair follicle tissue of the straight hair and the curved hair from the identical participant. In the curved hair, we found that adjacent hair matrix cells based on different hair germ fused to form a single hair follicle covered with an inner root sheath. That is, when a new hair cycle begins, multiple secondary hair germs fused by some factor, resulting in partial differences in the speed and timing of differentiation, resulting in distorted (asymmetric) hair shapes and acquired curved hair. Although this result is a study using a plucked hair follicle and does not actually show the condition in the living body, it is considered to be a new discovery to clarify one of the causes of acquired curved hair development. In addition, although the possibility remains purely speculative, we considered that the phenomenon observed this time is one factor of diffuse alopecia of woman. In the future, we would like to suppress the generation of acquired curved hair by examining the causes of fusion of multiple hair matrix cells.

Conflict of Interest Statement.

The authors declare that they have no conflict of interest.

References.

1. De la Mettrie R, Saint-Léger D, Loussouarn G, Garcel A, Porter C, Langaney A (2007) Shape variability and classification of human hair: a worldwide approach. Hum Biol 79:265-281.
2. Lindelöf B, Forslind B, Hedblad MA, Kaveus U (1988) Human hair form. Morphology revealed by light and scanning electron microscopy and computer aided three-dimensional reconstruction. Arch Dermatol 124:1359-1363.
3. Nagase S, Kajiura Y, Mamada A, Abe H, Shibuichi S, Satoh N, Itou T, Shinohara Y, Amemiya Y (2009) Changes in structure and geometric properties of human hair by aging. J Cosmet Sci 60:637-648.
4. Horio M, Kondo T (1953) Crimping of Wool Fibers. Text Res J 23:373-386.
5. Bryson WG, Harland DP, Caldwell JP, Vernon JA, Walls RJ, Woods JL, Nagase S,

Itou T, Koike K (2009) Cortical cell types and intermediate filament arrangements correlate with fiber curvature in Japanese human hair. J Struct Biol 166:46-58.

6. Nagase S, Tsuchiya M, Matsui T, Shibuichi S, Tsujimura H, Masukawa Y, Satoh N, Itou T, Koike K, Tsujii K (2008) Characterization of curved hair of Japanese women with reference to internal structures and amino acid composition. J Cosmet Sci 59:317-332.
7. S Thibaut, O Gaillard, P Bouhanna, DW Cannell, BA Bernard (2005) Human hair shape is programmed from the bulb. Br J Dermatol 152:632-638.
8. S Thibaut, P Barbarat, F Leroy, BA Bernard (2007) Human hair keratin network and curvature. Int J Dermatol Oct;46 Suppl 1:7-10.
9. Sriwiriyanont P, Hachiya A, Pickens WL, Moriwaki S, Kitahara T, Visscher MO, Kitzmiller WJ, Bello A, Takema Y, Kobinger GP (2011) Effects of IGF-binding protein 5 in dysregulating the shape of human hair. J Invest Dermatol 131:320-328.
10. G Cotsarelis, TT Sun, RM Lavker (1990) Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. Cell 29;61(7):1329-1337.
11. Fujimoto A, Farooq M, Fujikawa H, Inoue A, Ohyama M, Ehama R, Nakanishi J, Hagihara M, Iwabuchi T, Aoki J, Ito M, Shimomura Y (2012) A missense mutation within the helix initiation motif of the keratin K71 gene underlies autosomal dominant woolly hair/hypotrichosis. J. Invest. Dermatol 132:2342-2349.
12. PI Hynd, N. M Edwards, M Hebart, M McDowall, S Clark (2009) Wool fibre crimp is determined by mitotic asymmetry and position of final keratinisation and not ortho- and para-cortical cell segmentation. Animal 3:838-843.
13. Miteva M, Tosti A (2014) Pathologic diagnosis of central centrifugal cicatricial alopecia on horizontal sections. Am J Dermatopathol 36:859-864.
14. Herskovitz I, Miteva M (2016) Central centrifugal cicatricial alopecia: challenges and solutions. Clin Cosmet Investig Dermatol 17:175-181.
15. H Pinkus (1951) Multiple Hairs (Flemming-Giovannini): Report of Two Cases of Pili Multigemini and Discussion of Some Other Anomalies of the Pilary Complex. J Invest Dermatol 17:291-301.
16. Rachel S, Michael R (2012) Mesenchymal-epithelial interactions during hair follicle

morphogenesis and cycling. Semin Cell Dev Biol 23:917-927.

17. John T Headington (1984) Transverse Microscopic Anatomy of the Human Scalp. Arch Dermatol 120:449-456.
18. R Crowe, D Henrique, D Ish-Horowicz, L Niswander (1998) A new role for Notch and Delta in cell fate decisions: patterning the feather array. Development 125:767-775.
19. Favier B, Fliniaux I, Thélu J, Viallet JP, Demarchez M, Jahoda CA, Dhouailly D (2000) Localisation of members of the notch system and the differentiation of vibrissa hair follicles: Receptors, ligands, and fringe modulators. Devel Dynamics 218:426-437.
20. Iwasaki M, Kuroda J, Kawakami K, Wada H (2018) Epidermal regulation of bone morphogenesis through the development and regeneration of osteoblasts in the zebrafish scale. Developmental Biology 437:105-109.