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SHORT COMMUNICATION

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Human hair growth ex vivo is correlated with in vivo hair growth: selective categorization of hair follicles for more reliable hair follicle organ culture

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Abstract Of the numerous assays used to assess hair growth, hair follicle organ culture model is one of the most popular and powerful in vitro systems. Changes in hair growth are commonly employed as a measurement of follicular activity. Hair cycle stage of mouse vibrissa follicles in vivo is known to determine subsequent hair growth and follicle behavior in vitro and it is recommended that follicles be taken at precisely the same cyclic stage. This study was performed to evaluate whether categorization of human hair follicles by the growth in vivo could be used to select follicles of the defined anagen stage for more consistent culture. Occipital scalp samples were obtained from three subjects, 2 weeks later after hair bleaching. Hair growth and follicle length of isolated anagen VI follicles were measured under a videomicroscope. Follicles were categorized into four groups according to hair growth and some were cultured ex vivo for 6 days. Follicles showed considerable variations with respect to hair growth and follicle length; however, these two variables were relatively well correlated. Hair growth in culture was closely related with hair growth rate in vivo. Moreover, minoxidil uniquely demonstrated a significant increase of hair growth in categorized hair follicles assumed at a similar early anagen VI stage of hair cycle. Selection of follicles at a defined stage based on hair-growth rate would permit a more reliable outcome in human hair follicle organ culture.

Keywords Hair follicle organ culture · Hair growth · Hair cycle

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Since methods for the isolation and in vitro culture of human hair follicles were first established [2, 6], the hair follicle organ culture model has been the most popular and successful in vitro model for hair biology research [8]. Nevertheless, there are two weak points in this model, namely, difficulties associated with the isolation of hair follicles intact within several hours after removal exempt a hardship in obtaining sufficient human samples, and the considerable variability in hair-growth rate shown by anagen hair follicles [8].

Hair cycle stage of mouse vibrissa follicles in vivo is known to determine subsequent hair growth and follicle behavior in vitro and it is recommended that follicles should be taken at precisely the same cyclic stage [10]. Moreover, anagen rat vibrissa follicles retain cyclical activity in vitro and undergo a significant proportion of their growth cycle compared with in vivo age-equivalent littermates [7].

Human hair follicles undergo a growth cycle consisting of four distinct stages, namely, an active growth stage (anagen), a regressive stage (catagen), a resting stage (telogen), and a shedding stage (exogen). Uniquely, the anagen stage of human scalp hair follicles is known to last for up to 3–8 years [11]. Variabilities in the lengths of hair produced and in the durations of growth of human anagen hair follicles in vitro may arise partly from the use of anagen VI hair follicles in different substages (early-mid-late).

We present the results of a study that demonstrates that the hair growth of terminal human anagen VI hair follicles at extraction is correlated with their subsequent growth in vitro.

Three healthy volunteers, 52- and 54-year-old females (subjects 1 and 2) and one 31-year-old male (subject 3), were recruited for this study. None had experienced an episode of hair thinning or hair loss, recent illness, or general health disturbance, or showed evidence of a hair disorder by clinical examination. The Institutional Review Board of Seoul National University Hospital approved all procedures used in this study, and written informed consents were obtained from each subject.

Hairs of about 0.5×2 cm in size from an elliptical area in the occipital region over 2 cm from the occipital protuberance of each subject were clipped with scissors. A commercial bleaching agent (OBS Power Bleach®, OBS Co., Korea) was carefully treated onto the clipped hairs immediately after clipping (Fig. 1a, b). Two weeks later, excisional biopsies were performed (Fig. 1c), and tissue samples containing bleached hair follicles were cautiously dissected into single hair follicles. The isolation of anagen hair follicles was achieved using a surgical blade, a microscissors, and a watchmaker's forceps under a stereo dissecting microscope. As in the micrograft preparation for the follicular unit hair transplantation procedure, each follicle was isolated intact as a whole length of the follicle [9]. Any follicle visibly damaged during dissection was discarded.

Stereo images (20 times) of individual hair follicles were obtained using a non-contact video microscope system composed of a fixed SONY super HAD CCD™ camera and a monitor (iCamscope® ICS-305A, Sometech Co., Korea). Follicles that showed any follicular bulb abnormality, no growth, or retarded passive movement of hair shaft (indicative of a follicle in the telogen or catagen state) were excluded. The hair growth over 2 weeks and follicle length of each follicle were measured using the auto ruler software (IT-Pro3.0 Image Tracer Professional®, Sometech Co., Korea); hair growth (G) was defined as new black-hair production from the top of the epidermis to the bleached end of the hair, and the follicle length (L) was defined as the distance from the surface of epidermis to the base of the dermal papilla (Fig. 1d). According to the 2-week hair growth, anagen VI hair follicles were categorized into four groups: those showing a growth of –5.0 mm (group A), a growth of > 5.0–6.0 mm (group B), a growth of > 6.0–7.0 mm (group C), and a growth of > 7.0–8.0 mm (group D) (Fig. 1e).

From the defined four groups, some of the isolated hair follicles from three volunteers were combined together and assigned to hair follicle organ culture; group A ($N = 15$ follicles, five follicles per subject), group B ($N = 14$), group C ($N = 14$), and group D ($N = 11$). Whole hair follicles from each group were placed in Williams E medium (Gibco BRL, Gaithersburg, MD, USA) containing 10 ng/ml hydrocortisone, 10 μg/ml insulin, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin at 37°C in 5% CO_2 atmosphere. Supplemented medium was prepared just before experiment and replaced every other day during the 6-day period.

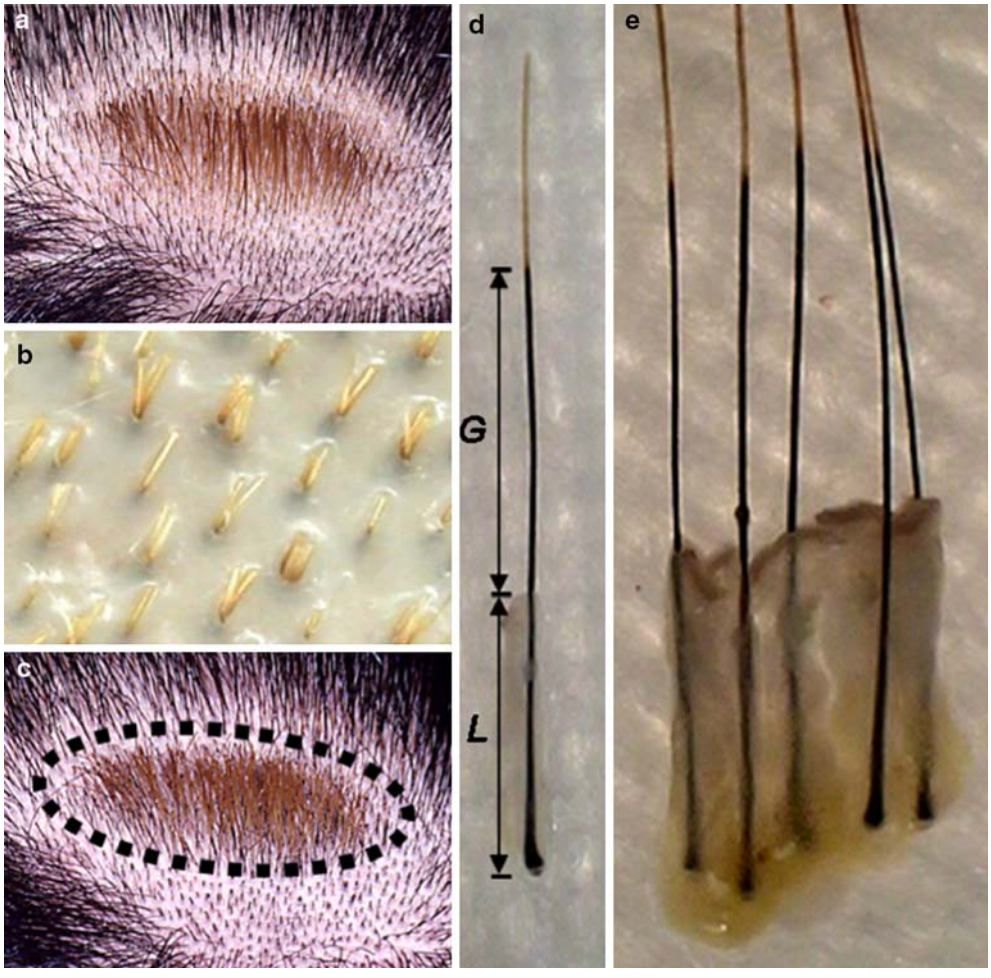

Fig. 1 Assignment of human anagen VI hair follicles by hair growth. **a** Occipital scalp hairs in an area of about 0.5×2 cm were treated with bleaching agent after clipping. **b** Macroscopic image (15 times) of the bleached area. **c** Two weeks later, scalp areas with bleached hairs were excised. **d** Magnified stereo image (20 times) of isolated anagen VI hair follicle. Hair growth (G) and follicle length (L) were measured with auto ruler software. **e** Isolated hair follicles were categorized according to 2-week hair growths

To validate the usefulness of selective categorization for human hair follicle organ culture, minoxidil was chosen as a standard test compound and evaluated whether to reproduce the in vivo hair-growth promotion in our system. Additionally, occipital hair follicles from another two 25-year-old male donors were categorized as above and incubated with 10 μM minoxidil (Sigma, St Louis, MO, USA) or 0.01% DMSO vehicle in phenol-tree William's E medium with ciprofloxacin (10 μg/ml) instead of streptomycin otherwise as described previously [1, 3].

Follicles were maintained free floating in individual wells of 24-well multiwell plates. The length of each follicle was measured on day 0 and every other day until day 6 after initiating culture at a magnification of 40 times, using an Olympus stereomicroscope with a calibrated eyepiece graticule.

The data obtained were statistically analyzed using Pearson's correlation analysis between 2-week hair growth and follicle length. Hair growth of ex vivo culture was compared between groups using repeated measures ANOVA and the effect of minoxidil treatment in each selected experimental group was determined using a *t*-test with correction for multiple comparisons. A P value of less than 0.05 was considered statistically significant.

Mean hair growths and follicle lengths of anagen VI follicles from three volunteers were 5.93 ± 0.75 and 5.09 ± 0.38 mm, respectively. However, anagen VI hair follicles diversely produced hair fibers of length from 3.83 to 7.70 mm over 2 weeks, although hair growths primarily fell in the ranges >5.0–6.0 mm (92 follicles, 41.1%) and >6.0–7.0 mm (85 follicles, 37.9%). Two subjects (subject 2 and 3) showed a peak in the former range (47.1%, 42.2%, respectively), whereas more than 50% of the hairs produced by the remaining volunteer (subject 1) fell in the latter range (57.5%, 23 of 40 follicles) (Fig. 2a).

Follicle length ranged from 3.74 to 6.17 mm; 52.7% (118 follicles) of anagen follicles were located over 5.0–5.5 mm beneath the epidermal surface, followed by >4.5 to 4.5 mm (66 follicles, 29.5%) and >5.5 mm (20 follicles, 8.9%). Similar to hair growth, follicle length revealed inter-individual variation. Follicle length in two volunteers (subjects 2 and 3) predominantly fell in the range >5.0–5.5 mm (58.7%), and 21 of 40 follicles from subject 1 were primarily located in the range >4.5–5.0 mm (Fig. 2b).

Follicle length presents a straightforward means of classifying the stage of a hair follicle [5]. We investigated the relationships between hair growth and follicle length in human samples. In general, statistically significant

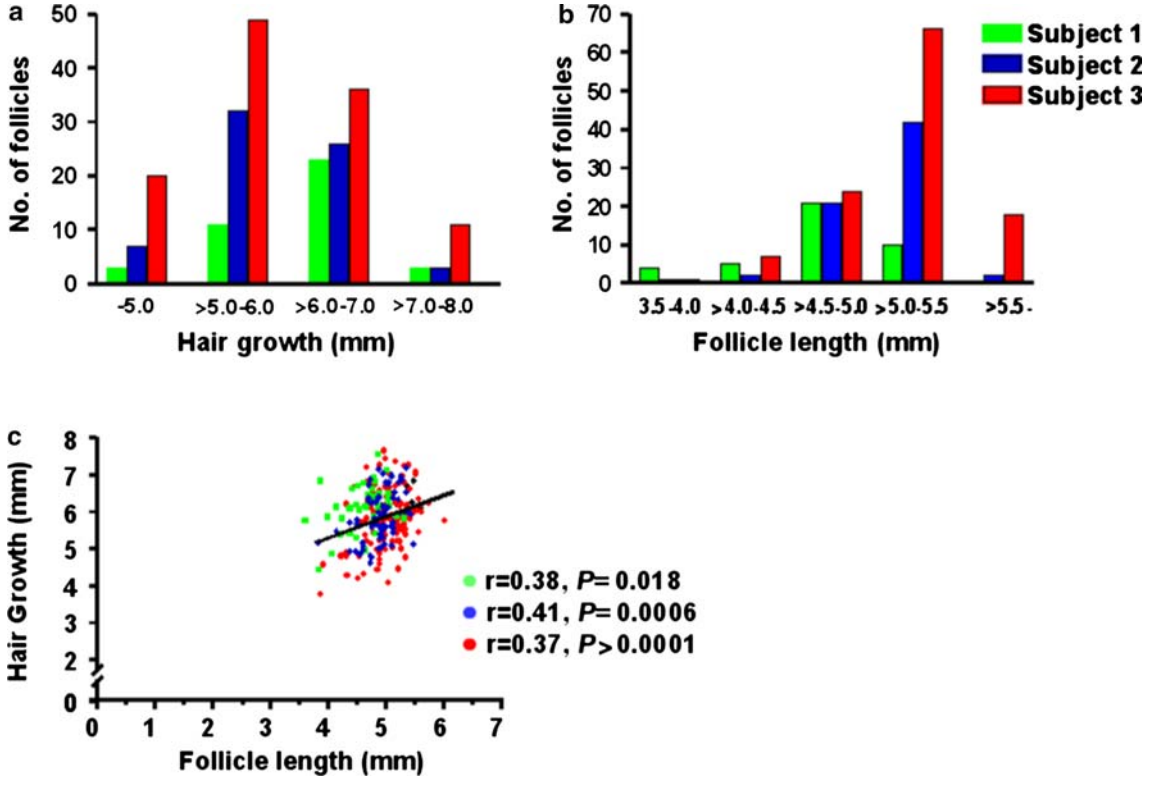

Fig. 2 Distributions of human anagen VI terminal hair follicles with respect to 2-week hair growth (**a**) and follicle length (**b**). Hair follicles showed variable hair growth and follicle length. Inter-individual variations in the distributions of these two variables were also observed for the three subjects. **c** The relationship between 2-week hair growth in vivo and follicle length. Significant correlations between these two variables were found for individual volunteers. Number of hair follicles: subject 1($N=40$); subject 2($N=68$); subject 3($N=116$)

correlations were found between 2-week hair growths and follicle lengths in each of the three volunteers (Fig. 2c).

A six-day culture of human hair follicles demonstrated that the hair-growth rate ex vivo consistently associated with hair growth in vivo (Fig. 3a). Within defined four groups of anagen VI follicles, a clear hierarchy of growth rate was evident in terms of the lengths of the hair fibers produced, that is, group D follicles demonstrated the highest average rate (0.25 mm/day), followed by group C (0.22 mm/day), and then group B (0.20 mm/day). Compared with the group A, hair follicles of group D showed a significant increase in the mean lengths of hairs produced on days 4 and 6 ($P < 0.05$).

In the experiment with minoxidil (Fig. 3b), 10 μM minoxidil significantly stimulated the hair growth of group D hair follicles after the six-day culture ($P < 0.05$), but showed no significant effect on the other three groups. Moreover, hair follicles of group D elongated longer and more consistently compared with other groups. Minoxidil-treated hair follicles of group D showed a greater increase in hair growth compared with those of group A ($P = 0.06$). Divided into two categories, minoxidil increased the hair growth of follicles of groups C and D greater than that of groups A and B ($P = 0.054$).

Among terminal human anagen VI follicles, a considerable variation was observed in hair growth and follicle length. It is uncertain whether these variations were due to the different anagen sub-stages of the hair follicles or to the innate characteristics of individual hair follicles.

Robinson et al. [10] demonstrated that murine vibrissae follicles in early anagen stage have a higher growth rate and a longer duration of hair production, and that this is followed by mid anagen and finally late anagen follicles. In a study by Van Neste and Demortier [13], who monitored human hair cycle transitions in vivo using contrast enhanced phototrichogram, growth rates

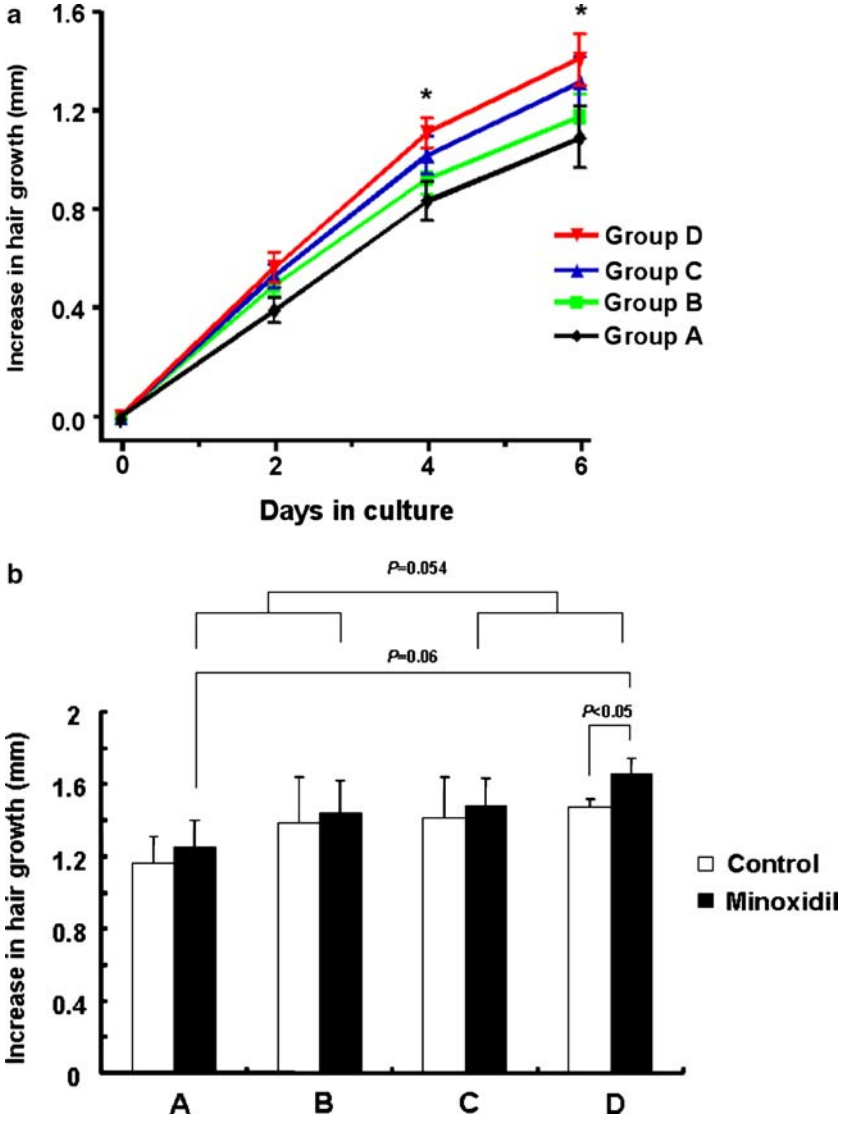

Fig. 3 **a** Ex vivo hair growth in hair follicle organ culture was closely related to in vivo hair growth. The hair follicles were selected based on hair growth for ex vivo culture from each of the three subjects: group A ($N = 15$, $L \leq 5.0$ mm), group B ($N = 14$, < 5.0 mm $L \leq 6.0$ mm), group C ($N = 14$, < 6.0 mm $L \leq 7.0$ mm) and group D ($N = 11$, < 7.0 mm $L \leq 8.0$ mm). *: $P < 0.05$ versus group A. **b** Effect of minoxidil on hair growth in ex vivo culture for 6 days. Anagen VI hair follicles were incubated in phenol-free William's E medium. Increased hair growth was observed only in group D hair follicles cultured at 10 μM minoxidil compared with the vehicle-treated control. Grouping A to D is described herein. Data are based on two 25-year-old male hair follicle donors using 30–40 follicles per each eight condition. Increases in hair growths are described as mean ± SEM

varies during the anagen stage, and they suggested that hair cycling is a reproducible process with a gradual and progressive regression. Anagen hair follicles are speculated to display different productivity programs as they transit from the early growth stage into the mature stage and finally into the regression stage, which shows a significant decline in the growth rate that announces transit into the catagen stage. Moreover, human hair follicles in the early anagen state are also known to show unusually high growth rates [4].

Murine hair follicles demonstrate a regular hair-growth cycle and a short anagen phase of about 2–3 weeks after depilation. The murine follicles are known to grow down during the anagen stage and to reach maximal length at anagen VI stage, and to retain this length until the catagen I and II stages. Thus, during anagen VI to catagen II cutaneous levels, where the dermal papillae are located, are similar [7]. However, in the present study, hair growth was relatively well correlated to follicle length. From our results, and considering a long human anagen stage of over 3 years, we hypothesize that human anagen VI hair follicles may achieve maximal length in the early anagen VI stage and then show a subtle but gradual ascent during the mid and late anagen stages before finally transiting into the catagen stage.

Previously, minoxidil was reported not to show a significant increase in ex vivo culture of human anagen VI hair follicles obtained from occipital scalp, which is different from in vivo hair growth promoting effect of minoxidil [3]. One of the causes of the disparity was speculated by the use of already maximally grown anagen VI follicles, because in vivo stimulation by minoxidil appears to target telogen and early anagen hair follicles [3, 12]. In our study, minoxidil uniquely demonstrated a significant increase of hair growth in group D hair follicles, which were considered as early anagen VI follicles. These findings also support our speculation that longer hair follicles with higher growth rates would be early anagen VI follicles.

In hair follicle organ culture experiments, change in hair growth is a commonly used parameter to measure follicular activity. As the hair growth characteristics of murine follicles, e.g., hair growth rate and duration of hair production, are dependent on stage at dissection, it is recommended to take follicles at the same closely defined stage of the hair cycle, and with respect to the rodent vibrissa follicle model, early anagen follicles were proposed to be best for property evaluations [10]. Similar to that found in rodent models, the present study showed that human hair growth in vivo correlated to the subsequent hair growth in organ culture.

In our study, by using the hair-bleaching method as a means of identifying new hair growth, we were able to determine the hair growth of each hair follicle. Before obtaining scalp hair samples for hair follicle organ culture, this technique could be usefully used to identify anagen VI follicles at a similar stage of the anagen cycle, which would improve result reliability and consistency. In addition, the follicle length was found to correlate to hair growth. The categorization of hair follicles by follicle length could be also a convenient means of choosing hair follicles in similar stages.

In conclusion, human hair follicles in anagen VI stage showed a considerable variation in hair growth and follicle length, which were relatively well correlated. Hair growth in vitro was found to be closely related with in vivo follicle activity. The selection of hair follicles at the same stage based on in vivo hair growth would permit a more reliable outcome in experiments involving human hair follicle organ culture.

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