



# Topical application of *Polygonum multiflorum* extract induces hair growth of resting hair follicles through upregulating Shh and $\beta$ -catenin expression in C57BL/6 mice

Hye-Jin Park<sup>a,b,\*</sup>, Nannan Zhang<sup>a</sup>, Dong Ki Park<sup>a,b</sup>

<sup>a</sup> Department of Bioscience and Biotechnology, Konkuk University, 1 Hwayang-dong, Kwangjin-gu, Seoul 143-701, Republic of Korea

<sup>b</sup> Division of Bioscience and Biotechnology, Bio/Molecular Informatics Center, Konkuk University, Seoul 143-701, Republic of Korea

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## ABSTRACT

**Ethnopharmacological relevance:** *Polygonum multiflorum* has traditionally been used for treating patients suffering from baldness and hair loss in East Asia.

**Aim of the study:** The present study sought to investigate the hair growth promoting activities of *Polygonum multiflorum* and its mechanism of action.

**Materials and methods:** The *Polygonum multiflorum* extract was topically applied to the shaved dorsal skin of telogenic C57BL6/N mice. To determine the effect of *Polygonum multiflorum* extract in telogen to anagen transition, the expression of  $\beta$ -catenin and Sonic hedgehog (Shh) was determined by immunohistochemistry analysis.

**Results:** *Polygonum multiflorum* extract promoted hair growth by inducing anagen phase in telogenic C57BL6/N mice. In *Polygonum multiflorum* extract treated group, we observed increase in the number and the size of hair follicles that are considered as evidence for anagen phase induction. Immunohistochemical analysis revealed that earlier induction of  $\beta$ -catenin and Shh were observed in *Polygonum multiflorum* extract treated group compared to that in control group.

**Conclusion:** These results suggest that *Polygonum multiflorum* extract promotes hair growth by inducing anagen phase in resting hair follicles.

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## 1. Introduction

Hair is a protective appendage on the body that is considered accessory structure of the integument along with sebaceous glands, sweat glands and nails (Ebling, 1987). A unique characteristic of hair growth is its cyclicity, which involves a growth phase (anagen), an involution phase (catagen) and a resting phase (telogen phase) (Paus et al., 1999).

Hair loss is an emotionally distressing disease in human. Further, many human diseases are associated with hair loss. There are many causes of hair loss in men and women, including disease, nutritional deficiency, aging, hormone imbalance, and stress. For instance, a genetically predisposed person, whose hair follicles are continuously exposed to dihydrotestosterone (DHT), has a shorter anagen phase (growth phase of hair). When anagen phase becomes gradually briefer and briefer, hairs become finer, shorter and less deeply colored, which leads to hair loss. Many hair promoting agents are focused in inducing anagen from telogenic hairs.

The estimated annual market value for anti-hair loss is multi-billion dollars. Despite the plethora of 'anti-hair loss' agents, convincing evidence-based medicine still is the exception rather than the rule in the market. To date, just two anti-hair loss drugs (i.g. the dihydrotestosterone-suppressing 5 $\alpha$ -reductase inhibitor, finasteride and the anti-hypertensive potassium channel opener, minoxidil) have been approved by FDA (D'Amico and Roehrborn, 2007). Conventional anti-hair loss drugs such as finasteride and minoxidil limit their therapeutic uses due to their undesirable side-effects and low cure rate.

Given the limited, transient and somewhat unpredictable efficacy of these approved anti-hair loss medicines, it is important to develop novel pharmacological treatments and agents. There are many attempts being focused to discover more effective materials from traditional herbal medicines that prevent hair loss. For instance, *Polygonum Multiflorum* Thunb (Polygonaceae), known as Fo-Ti, has been traditionally used to treat patients suffering from baldness and hair loss in East Asia (Rector- Page, 1992). Several companies produce hair growth promoting agents, containing the extract of *Polygonum multiflorum*. Recent study demonstrated that the active component 2,3,5,4'-tetrahydroxystilbene-2-O-beta-D-glucoside (THSG) from *Polygonum multiflorum* induced melanogenesis in melanocytes

\* Corresponding author at: Department of Bioscience and Biotechnology, Konkuk University, 1 Hwayang-dong, Kwangjin-gu, Seoul 143-701, Republic of Korea. Tel.: +82 2 455 5589; fax: +82 2 455 5517.

E-mail address: [hyejinpark@gmail.com](mailto:hyejinpark@gmail.com) (H.-J. Park).

(Jiang et al., 2009). However, the hair growth promoting activity of *Polygonum multiflorum* has not scientifically proven yet. In this study, we investigated the hair growth promoting activities of *Polygonum multiflorum* and its mechanism of action.

## 2. Materials and methods

### 2.1. Materials

The immunocruz staining system kit (Santa cruz Biotechnology, Santa cruz, CA, USA) and DAB chromogen kit (Vector laboratories, Burlingame, CA, USA) were purchased. Anti- $\beta$ -catenin (Cell signaling technology, Danver, MA, USA) and anti-Shh (Abnova, Taipei, Taiwan) antibodies were purchased. Hematoxylin (BBC Biochemical, Mount Vernon, WA, USA) and eosin (Shimakyu's Pure Chemicals, Osaka, Japan) were purchased.

### 2.2. Preparation and fermentation of *Polygonum multiflorum* extracts

The dried, powdered leaves of *Polygonum multiflorum* were provided by Cell Activation Research Institute (CARI, Seoul, Korea). Authenticated voucher specimen of *Polygonum multiflorum* (Kucari 1002) is deposited in the Herbarium at College of Bioscience and Biotechnology, Konkuk University (Seoul, Korea). In brief, water extracts of *Polygonum multiflorum* were prepared in 850 ml autoclaved deionized water for 5 min, followed by the addition of *Lactobacillus sp.* *Polygonum multiflorum* (PM) containing *Lactobacillus sp.* was fermented at 37 °C. After fermentation, olive oil was added to the *Polygonum multiflorum* (PM) containing *Lactobacillus sp.*, which was fermented for 45 days. The dried, powdered PM containing *Lactobacillus sp.* was boiled in hot water (2000 ml) for 2 h, and the hot-water extract was chilled, filtrated through Advantech No. 2 filter paper (Osaka, Japan) and evaporated to dryness.

### 2.3. Experimental animals

Healthy C57BL6/N mice (6-week-old, 5 mice per group) were obtained from Orient Bio (Eumsung, Korea). Mice were cared in a controlled barrier facility within College of Veterinary Medicine, Konkuk University. Temperature ( $23 \pm 2$  °C), humidity (35–60%),

and photoperiod (12 h light:12 h darkness cycle) were kept constantly. The animal study was performed in accordance with the institutional guidelines (The Institutional Animal Care and Use Committee (IACUC) at Konkuk University (Seoul, Korea)). The authorization code number from IACUC is ku09048.

### 2.4. Experimental studies with *Polygonum multiflorum* extract

Ten animals in 2 randomized groups ( $n=5$ ) were used for the study of hair promoting activity. All animals were shaved using an animal clipper at 6 weeks of age, at which all hair follicles were synchronized in the telogen stage. *Polygonum multiflorum* extract (4.7 mg/12 cm<sup>2</sup>) or vehicle (water containing *Lactobacillus sp.*) was applied topically on dorsal skin of C57BL/6N mice using swaps. At each 1, 2, 3 and 4 week, one mouse from groups 1 and 2 was sacrificed to obtain skin specimen. Visible hair growth was recorded at each 1, 2, 3 and 4 week.

### 2.5. Histological studies

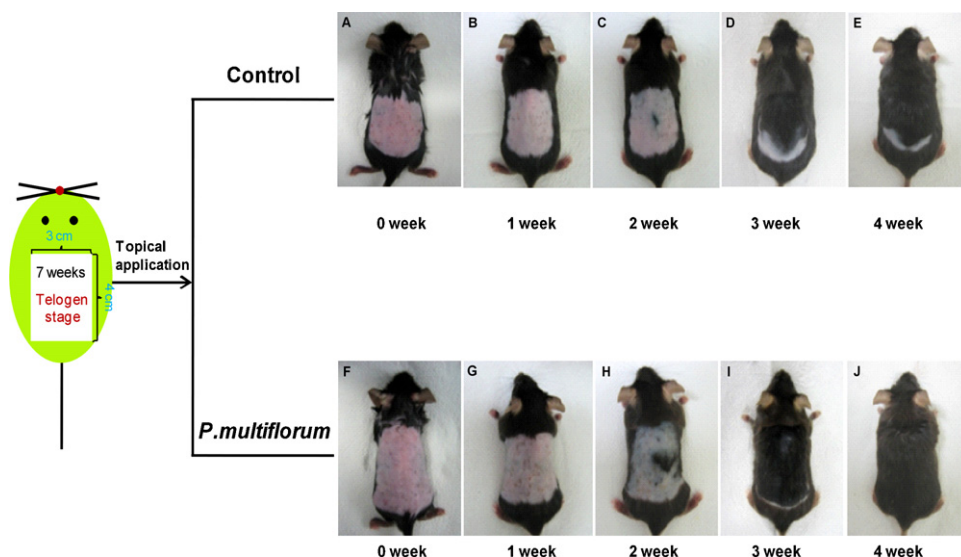
12 cm<sup>2</sup> of dorsal skin was excised between fore and hind legs after topical application with *Polygonum multiflorum* extract (4.7 mg/12 cm<sup>2</sup>) at the indicated time points. Dorsal skin was maintained in 10% neutral formalin and embedded in paraffin blocks to obtain longitudinal and transverse section. 5  $\mu$ m sections were stained with hematoxylin and eosin (H&E). Digital photomicrographs were taken from representative areas at a fixed magnification of 100 $\times$ .

### 2.6. Hair follicle count

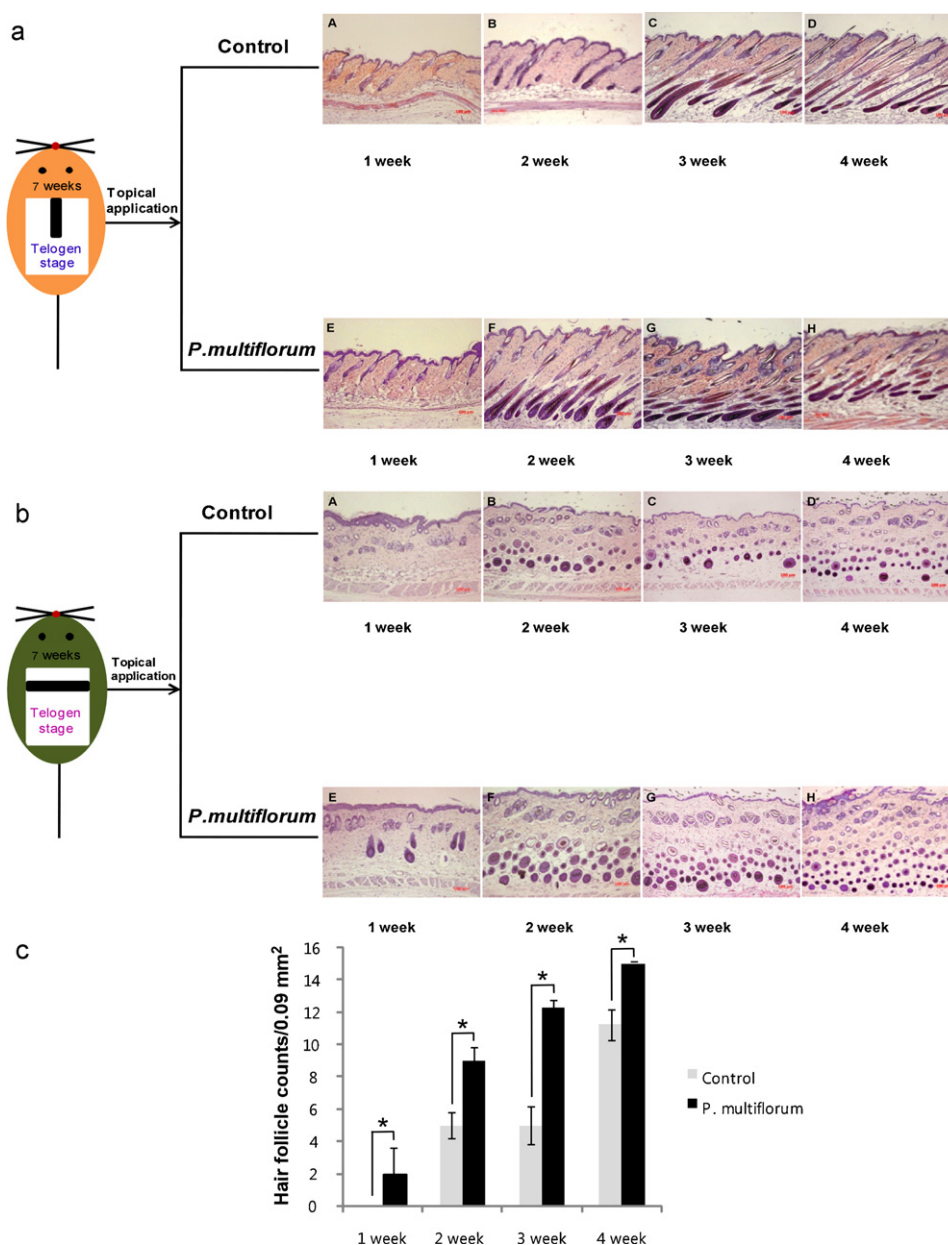
The H&E stained slides were photographed using a digital photomicrograph and all of the images were cropped in a fixed area of 300 pixels width. We counted hair follicles manually in a fixed area (0.09 mm<sup>2</sup>). Digital photomicrographs were taken from representative areas at a fixed magnification of 100 $\times$ .

### 2.7. Hair length determination

Hairs were plucked randomly from shaved dorsal area at 0, 1, 2, 3 and 4 week. After plucking 10 hairs per mouse, we measured the average hair length manually.



**Fig. 1.** Hair growth promoting effect of *Polygonum multiflorum* extract. Telogen-matched, 7-week old C57BL6/N mice were shaved and topically applied with vehicle ( $n=5$ ) or *Polygonum multiflorum* extract (4.7 mg/12 cm<sup>2</sup>) ( $n=5$ ). Photographs were taken every week after applying *Polygonum multiflorum* extract or vehicle on the shaved dorsal skin. More black coloration was observed in *Polygonum multiflorum* extract treated mice than in control. Control (A–E); *Polygonum multiflorum* extract (F–J).



**Fig. 2.** *Polygonum multiflorum* extract increased the size and number of hair follicles. The effect of *Polygonum multiflorum* extract on the hair follicles was analyzed using hematoxylin–eosin (H&E) staining. C57/BL6 mice treated with vehicle and *Polygonum multiflorum* extract at concentration of 4.7 mg/12 cm<sup>2</sup> (a) longitudinal sections of the dorsal skins (0, 1, 2, 3 and 4 weeks) were stained using H&E. (b) Transverse sections of the dorsal skins (0, 1, 2, 3 and 4 weeks) were stained using H&E. (c) The number of hair follicles in deep subcutis. The image shown is the representative picture of 5 mice. Bars: 100  $\mu$ m. Control (A–D); *Polygonum multiflorum* extract (E–H).

## 2.8. Immunohistochemistry

Dorsal skins were stained with anti- $\beta$ -catenin and Shh antibodies which are evidence of anagen induction. To quench endogenous peroxidase activity, deparaffinized sections were pretreated with 1% peroxidase for 10 min. After washing with PBS, the sections were incubated with serum to block non-specific binding of biotinylated secondary antibody for 40 min and then incubated with anti- $\beta$ -catenin and Shh antibodies for 2 h. Slides were incubated with biotinylated secondary antibody for 30 min. After incubating with HRP–streptavidin complex to detect secondary antibody for 30 min, slides were developed until light brown staining was visible with DAB chromogen kit. The slides were counterstained with 1% methyl green for 1 min.

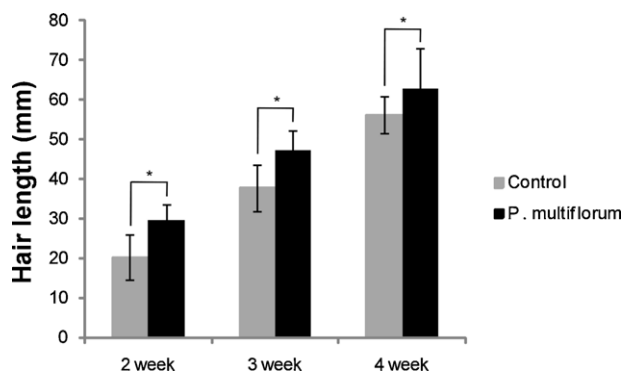
## 2.9. Statistical analysis

Results are expressed as mean  $\pm$  SD. Student's *t*-test or One-way ANOVA/Dunnnett's *t*-test was used for assessment of significance between controls and treatments. Statistical analysis was performed using SPSS, version 12 (SPSS Inc., Chicago, IL, USA).

## 3. Results

### 3.1. The effect of *Polygonum multiflorum* extract on hair growth

The black pigmentation was taken as evidence for transition of hair follicles from telogen to anagen phase (Peters et al., 2002). To evaluate the hair growth activity of *Polygonum multiflorum*, we topi-

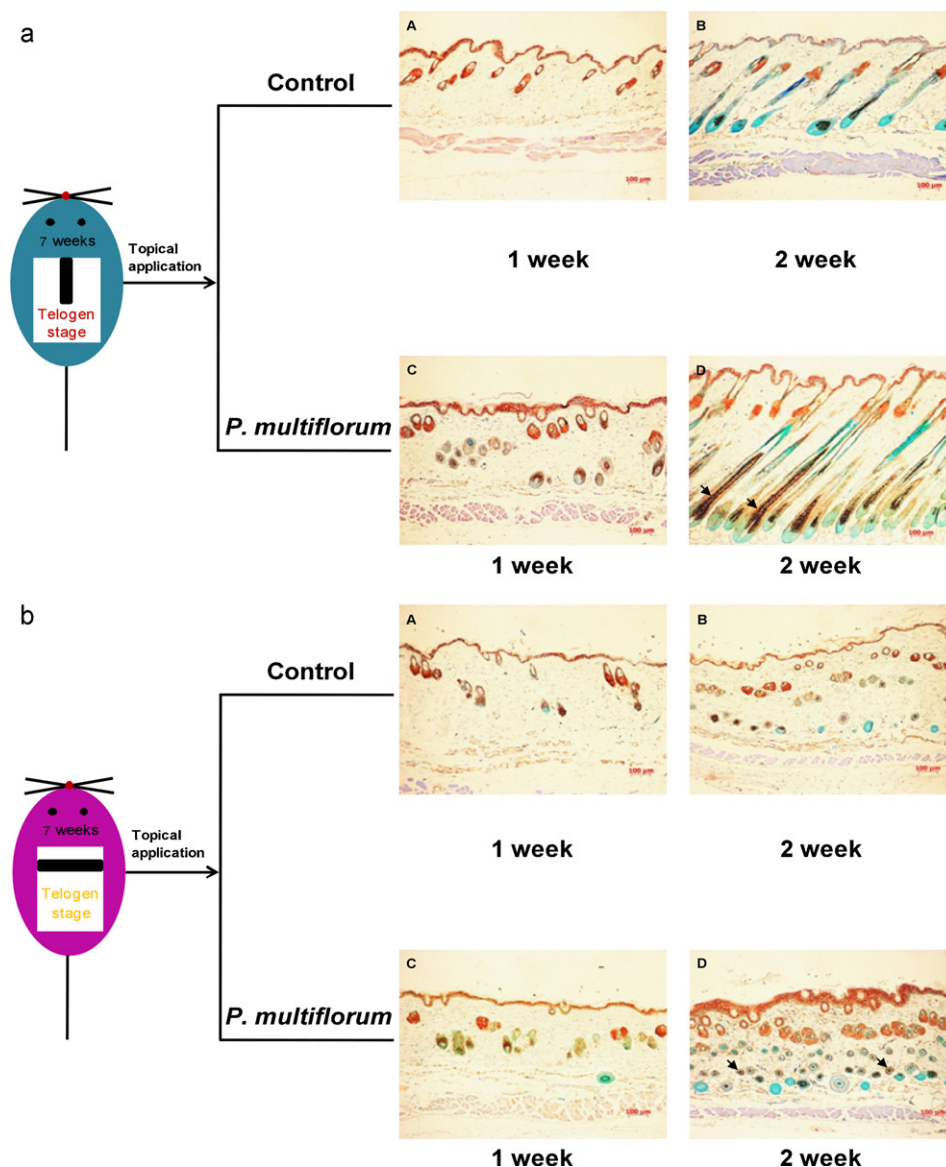


**Fig. 3.** Hair length at each week after topical application of *Polygonum multiflorum* extracts. The length of randomly plucked hairs ( $n=10$ ) was measured at different time intervals (2, 3 and 4 weeks) after topical application of *Polygonum multiflorum* extract. Data shown represent means  $\pm$  standard deviation (S.D.) of three independent experiments. One-way ANOVA was used for comparisons of multiple group means followed by Dunnett's  $t$ -test. ( $p < vs.$  control).

cally applied *Polygonum multiflorum* extract ( $4.7 \text{ mg}/12 \text{ cm}^2$ ) on the shaved dorsal skin of telogenic C57BL/6 mice for 1, 2, 3, and 4 weeks. Each week we evaluated the degree of hair growth by observing the skin color. At 2 week, we observed *Polygonum multiflorum* extract induced black coloration in the shaved skin of telogenic C57BL/6 mice, while neither less visible hair growth nor black coloration was observed in control group (Fig. 1). At 4 week, we observed that hair growth from control group was confined to the proximal parts of epidermis, emphasizing its dormancy, while *Polygonum multiflorum* extract treated group showed overall hair growth which was not confined to the proximal parts (Fig. 1).

### 3.2. Effect of *Polygonum multiflorum* extract on hair follicle number and hair shaft elongation

Increase in the number and the size of hair follicles was observed during anagen phase induction (Ogawa and Hattori, 1983). The anagen phase is associated with increase in the size and the number of hair follicles that lie in deep subcutis compared to that of telogen phase where hair follicles lie in dermis only (Datta et al.,



**Fig. 4.** Induction of  $\beta$ -catenin after topical application of *Polygonum multiflorum* extract. (a) Longitudinal sections of the dorsal skins (0, 1, 2, 3 and 4 weeks) from each group were stained for  $\beta$ -catenin by immunohistochemistry (brown staining). (b) Transverse sections of the dorsal skins (0, 1, 2, 3 and 4 weeks) from each group were stained for  $\beta$ -catenin by immunohistochemistry (brown staining). Bars:  $100 \mu\text{m}$ . Control (A–B); *Polygonum multiflorum* extract (C–D).

2009). In the representative longitudinal and transverse sections, we observed that the hair follicles in *Polygonum multiflorum* extract treated group appeared earlier than those in the control group (Fig. 2a and b). The number of hair follicles of the relative area ( $0.09 \text{ mm}^2$ ) in *Polygonum multiflorum* extract treated group was higher than in the control group (Fig. 2c). To confirm whether *Polygonum multiflorum* extract promoted hair growth, we measured the length of 10 hairs plucked from the dorsal skin of each mouse at 2, 3, and 4 week. Since visible hair shaft was observed after 2 weeks, we measured length of 2, 3, and 4 week-old hairs. As shown in Fig. 3, the length of hairs in *Polygonum multiflorum* extract treated group was significantly longer than that of control group ( $p$ -value  $< 0.05$ ).

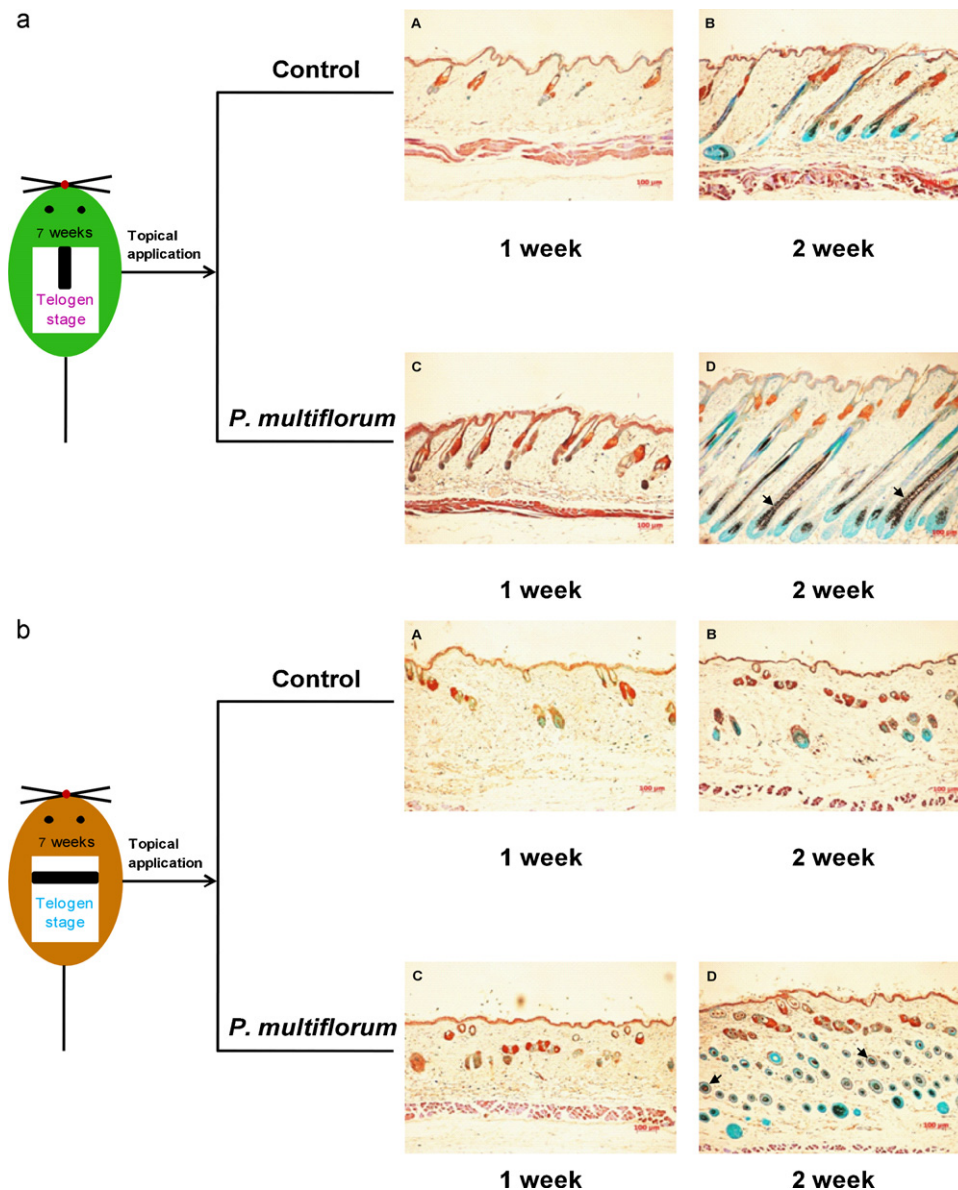
### 3.3. *Polygonum multiflorum* extract induced anagen phase from telogenic C57BL/6 mice

In order to elucidate the mechanism of the early events of anagen induction by *Polygonum multiflorum* extract, we measured the expression levels of  $\beta$ -catenin and Sonic hedgehog (Shh) by

immunohistochemistry analysis. Previously it was reported that transiently activated  $\beta$ -catenin induced the transition from telogen phase to anagen phase hair at the site of exposure (Hebert et al., 1994; Van Mater et al., 2003). We observed that  $\beta$ -catenin protein expression levels were higher in *Polygonum multiflorum* extract treated group than in control group at 2 weeks (Fig. 4). Sonic hedgehog (Shh) also serves as a key regulator of follicular growth and cycling by inducing the transition from the telogen to anagen phase (Stenn and Paus, 2001). Immunohistochemical analysis result showed that Shh expression was up-regulated in *Polygonum multiflorum* extract treated group compared to that in control group at 2 weeks (Fig. 5).

## 4. Discussion

The demands for hair growth promoting agents led to a multibillion-dollar industry. In Japan, approximately 60% of total Japanese male population is suffering from hair loss. The estimated market value is 4–5 billion dollars per year in Japan (Shimbun,



**Fig. 5.** Induction of Shh expression after topical application of *Polygonum multiflorum* extract. (a) Longitudinal sections of the dorsal skins (0, 1, 2, 3 and 4 weeks) from each group were stained for Shh by immunohistochemistry (brown staining). (b) Transverse sections of the dorsal skins (0, 1, 2, 3 and 4 weeks) from each group were stained for Shh by immunohistochemistry (brown staining). Bars: 100  $\mu\text{m}$ . Control (A–B); *Polygonum multiflorum* extract (C–D). Shh; Sonic hedgehog.

2005). Although hair loss disorders are not life-threatening, they are extremely emotionally distressing diseases which make afflicted patients vulnerable. Minoxidil is a popular hair growth promoting drug, but it is reported that it can cause adverse dermatological effects such as pruritis, dryness, scaling, local irritation, dermatitis (DeVilz, 1990). Finasteride is also a widely used hair growth promoting drug for androgenic alopecia patients, which is not recommended for female patients (Glina et al., 2004; Kaufman et al., 1998). It is reported that minoxidil is efficacious in promoting hair growth in patients with androgenic alopecia by inducing hair follicles to undergo transition from the early to late anagen phase (Gregoriou et al., 2010; Tsuboi et al., 2009). Conventional drugs such as finasteride and minoxidil limit their therapeutic uses due to their undesirable side-effects and low efficacy for treating hair loss or hair thinning. C57BL/6 mice are useful models for screening hair growth promoting agents, as their truncal pigmentation is dependent on their follicular melanocytes, producing pigment only during anagen (Plonka et al., 2005).

It is reported that THSG, an active compounds from *Polygonum multiflorum*, induced melanogenesis in melanocytes (Jiang et al., 2009), which suggested that it might promote hair growth by increasing anagen-phase hair follicles. In order to improve the bioavailability of *Polygonum multiflorum* extract, we produced *Polygonum multiflorum* extract after microbial fermentation using strains of *Lactobacillus*. Therefore, we investigated the hair growth promoting activity of the fermented *Polygonum multiflorum* extract using 7 week-old C57BL6/N mice which are in the stable telogen phase. The shaved back skins of C57BL6/N were treated with topical application of *Polygonum multiflorum* extract for 1, 2, 3, and 4 weeks. At 2 weeks, *Polygonum multiflorum* extract induced hair growth in the telogenic C57BL/6 mice, while neither less visible hair growth was observed in the control group. To further investigate the hair growth promoting effect, we plucked 10 hairs per mouse randomly from the treated area and measured the hair length. The hair length of *Polygonum multiflorum* extract treated mice was significantly longer than control group. Further, we will compare the hair promoting activity between *Polygonum multiflorum* water extract and fermented *Polygonum multiflorum*. If so, we will analyze the active compounds from each extract, which are responsible for the hair promoting activity.

Various hormones, growth factors and development-related molecules are involved in hair follicle growth (Boivin et al., 2006; Datta et al., 2009; Stenn and Paus, 2001; Yamazaki et al., 1999). To trigger anagen onset, several activators must be expressed up to a critical threshold concentration. Among them,  $\beta$ -catenin and Sonic hedgehog (Shh) expression play key regulators of hair follicular growth and cycling that act as anagen-inducing signaling molecules (Peters et al., 2002; Stenn and Paus, 2001). Induced  $\beta$ -catenin expression was observed in the dermal papilla at anagen onset and also detected in the stem cell progeny in the hair matrix throughout anagen phase (Schneider et al., 2009). Shh mainly expressed during anagen phase. When catagen phase of hair follicles begins, Shh expression ceases and its expression is hard to detect in the telogen hairs (Oro and Higgins, 2003). To elucidate the molecular mechanism of *Polygonum multiflorum* extract in inducing anagen hair follicles, we examined the expression levels of  $\beta$ -catenin and Sonic hedgehog (Shh) in the skin. Immunohistochemical analysis result showed that  $\beta$ -catenin and Shh expression were up-regulated in *Polygonum multiflorum* extract treated group compared to that in control group at 2 weeks. Some studies showed that continuous  $\beta$ -catenin signaling is required to maintain hair follicle tumors (Lo Celso et al., 2004). We observed that Shh and  $\beta$ -catenin expression levels gradually began to reduce in both groups after 3 week (data not shown), indicating that anagen phase of hair follicles was ceased (Datta et al., 2009). Further experiments are needed to identify active components in fermented *Polygonum multiflorum*

extracts and to determine their mechanisms of action, which might be responsible for the hair promoting activity.

In summary, it was reported for the first time that *Polygonum multiflorum* extract promoted hair growth by inducing anagen in telogenic C57BL6/N mice. In *Polygonum multiflorum* extract treated group, we observed an increase in the number and the size of hair follicles that is considered as evidence for anagen phase induction. Immunohistochemical analysis revealed that  $\beta$ -catenin and Shh were expressed earlier in *Polygonum multiflorum* extract treated group than that in control group. Taken together, these results suggest that *Polygonum multiflorum* extract promote hair growth by inducing anagen phase of hair follicles.

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