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ORIGINAL ARTICLE

Cutaneous application of α-methylspermidine activates the growth of resting hair follicles in mice

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Abstract Recent studies using transgenic animals have revealed a crucial role for polyamines in the development and the growth of skin and hair follicles. In mammals, the growth of hair is characterized by three main cyclic phases of transformation, including a rapid growth phase (anagen), an apoptosis-driven regression phase (catagen) and a relatively quiescent resting phase (telogen). The polyamine pool during the anagen phase is higher than in telogen and catagen phases. In this study, we used α-methylspermidine, a metabolically stable polyamine analog, to artificially elevate the polyamine pool during telogen. This manipulation was sufficient to induce hair growth in telogen phase mice after 2 weeks of daily topical application. The application site was characterized by typical features of anagen, such as pigmentation, growing hair follicles, proliferation of follicular keratinocytes and upregulation of β-catenin. The analog penetrated the protective epidermal layer of the skin and could be detected in dermis. The natural polyamines were partially replaced by the analog in the application site. However, the combined pool of natural spermidine and α-methylspermidine exceeded the physiological spermidine pool in telogen phase skin. These results highlight the role of polyamines in hair cycle regulation and show that it is possible to control the process of hair growth using physiologically stable polyamine analogs.

Keywords Alopecia · Polyamine · Skin · Spermidine

Abbreviations

α-MeSpd	α-Methylspermidine
DFMO	Difluoromethylornithine
ODC	Ornithine decarboxylase
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSAT	Spermidine/spermine N^1-acetyltransferase

Introduction

The hair follicle is a complex mini-organ that undergoes three distinct and life-long cyclic transformations including a rapid growth phase (anagen), a short apoptosis-driven regression phase (catagen) and a relatively long quiescent phase (telogen) (Cotsarelis 1997). During the anagen phase, keratinocytes in the hair bulb divide at a rapid rate with a mitotic index comparable to that of bone marrow and intestinal epithelium (Vanscott et al. 1963). Follicular keratinocytes soon stop proliferation and commence the catagen phase, which is characterized by apoptosis (Cotsarelis 1997). Several growth factors, such as keratinocyte growth factor (Danilenko et al. 1995), nerve growth factors (Botchkareva et al. 2000) and fibroblast growth factors (Hébert et al. 1994) have been demonstrated as mediators playing key roles in the hair cycle. However, the actual

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intracellular mechanisms that regulate the hair cycle are not yet fully comprehended (Alonso and Fuchs 2006).

Concentrations of epidermal polyamines change in a manner that correlates with hair cycling phases. We have demonstrated an increasing concentration of the natural polyamines namely putrescine, spermidine and spermine during morphogenesis of hair during the first 10 days of postnatal life of mice. The polyamine pool decreased to its lowest level at the time of first telogen at day 20 and peaked again at the time of first anagen at day 27 (Pietilä et al. 2001). The increased concentration of the natural polyamines in the rapidly proliferating keratinocytes of the anagen phase is not surprising, as many studies have demonstrated that a continuous and strictly regulated supply of polyamines is crucial for survival and proliferation of normal and cancerous cells (Bachrach 2004; Jänne et al. 2005). Furthermore, the expression of the biosynthetic enzyme of polyamine metabolism, ornithine decarboxylase (ODC) is upregulated in the entire length of the anagen phase hair follicle, whereas the expression is limited only to a small number of cells residing in the bulge region during telogen phase (Soler et al. 1996). The role of polyamines in the regulation of hair growth has been shown in two independent studies using transgenic animals. Both overexpression of either ODC (Soler et al. 1996) or spermidine/spermine-N^1-acetyltransferase (SSAT) (Pietilä et al. 1997) leads to cutaneous abnormalities, such as wrinkling of skin, abnormal hair follicles (dermal cyst) and loss of hair. It is surprising that the activation of both polyamine biosynthesis (overexpression of ODC) and catabolism (overexpression of SSAT) lead to similar phenotype. Both transgenic lines, however, show a massive overaccumulation of putrescine in the skin. The critical role of putrescine was demonstrated when these animals were treated with difluoromethylornithine (DFMO), a specific inhibitor of ODC. The treatment decreased the putrescine level in the skin to the extent that the cutaneous abnormalities described above were alleviated and the hair growth was triggered (Soler et al. 1996; Pietilä et al. 2005).

Hair cycle-associated fluctuation of polyamine concentrations and the skin abnormalities in the transgenic animals prompted us to investigate whether the growth of hair during the resting stage could be triggered by artificial elevation of polyamine pool. Anagen initiation and maintenance have been previously achieved by treating the telogen phase mice with cyclosporine A (Paus et al. 1989), platelet-derived growth factor (PDGF) (Tomita et al. 2006) or with a short peptide "GPIGS" derived from *Bacillus* sp. (Tsuruda et al. 2005). However, the natural polyamines being physiologically unstable are not feasible candidates for the proposed study. Recently synthesized α-methylated polyamine analogs have been proven to be metabolically stable and to serve as surrogate molecules by substituting their natural counterparts (Järvinen et al. 2005). Consequently, α-methylspermidine (α-MeSpd) has used as an appropriate analog to elucidate the specific role of polyamines in both in vivo and in vitro studies. For example, α-MeSpd treatment restores liver regeneration (Räsänen et al. 2002), reverses cytostasis in cultured fetal fibroblasts (Järvinen et al. 2005) and prevents acute pancreatitis triggered by depletion of polyamines (Hyvönen et al. 2006; Merentie et al. 2007).

Materials and methods

Experimental animals

C57BL/6 mouse strain has been described to be a well-suited animal model to investigate the underlining physiological events during the hair cycle phases due to occurrence of naturally synchronized hair cycle with cyclic pigmentation (Slominski et al. 1991). Hair of 7 weeks old (the approximate starting time for the second telogen) female C57BL/6 mice were shaved at the caudodorsal part of the skin and the animals were divided into three groups, the vehicle group comprising of five mice and the two treatment groups each comprising of six mice. On the shaved part of the skin, each animal in the first, second and the third group was treated with 5% (w/v) α-MeSpd (synthesized as described in Järvinen et al. 2006), 5% (w/v) spermidine (Sigma, St. Louis, MO) or vehicle (60% ethanol, 5% methyl cellulose and 20% propylene glycol) (Sigma) in 20 µl of the treatment volume, respectively. Animals were treated on 5 week days for three consecutive weeks. The animal experiments were approved by the provincial government of Southern Finland.

Cell culture

Fetal primary keratinocytes were isolated as described by previously (Caldelari et al. 2000). The keratinocytes were cultured in defined CnT-02 medium (CELLnTEC, Bern, Switzerland). Only cultures with a low passage number were used to avoid polyploidy that has been described in high-passage mouse keratinocyte cultures (Bruegel Sanchez et al. 2004). The effect of α-MeSpd on proliferation and differentiation of the cells was studied using thymidine incorporation assay. Cells were plated with or without 10 µM α-MeSpd in 6-well plates at a density of 20,000 cells/cm^2. On the following day, the calcium concentration was raised to 0.3 mM in three wells of each plate to initiate differentiation. For four consecutive days, the proliferation was analyzed from triplicate wells of each treatment group by incubating them with 1 µCi/ml [methyl ^{3}H] thymidine (82 Ci/mmol) (Amersham, GE Healthcare, Fairfield, CT) for 2 h followed by subsequent trypsinization and counting.

Histology and immunohistochemistry

Samples taken from the treated sites of the skin were fixed for 24 h with Histochoice (Amresco, Solon, OH). For detection of proliferating cell nuclear antigen (PCNA), the samples were fixed with buffered formalin (10%) solution for 24 h. Following the fixation, samples were washed with 0.1 M sodium phosphate buffer (pH 7.4), dehydrated in graded ethanol, embedded in the paraffin and cut into 5-µm thick longitudinal sections. The sections were stained with hematoxylin and eosin (H–E). For immunohistochemistry, rabbit polyclonal anti-PCNA (1:750) (PC-10, Santa Cruz Biotechnology, Santa Cruz, CA), anti-K6 (Covance, Princeton, NJ) and anti-BrdU-POD (Roche, Basel, Switzerland) were used. PCNA antigen retrieval was carried out by boiling samples in 0.01 M citric acid buffer pH 6.0. To visualize the rabbit polyclonal primary antibodies, we used Power Vision Poly-HRP Rabbit IgG IHC kit (Immunovision technologies, Leica Microsystems, Wetzlar, Germany).

Polyamine measurements

The skin samples taken from the treatment site were heated in water bath at 55°C for 20 s. Thereafter, dermis and epidermis were separated by scalpel blade with gentle scraping. Separated samples of dermis and epidermis were homogenized under Tris buffer (25 mM Tris, pH 7.4, 1 mM dithiothreitol, 0.1 mM EDTA) and the proteins were precipitated by 5% sulfosalicylic acid (w/v final concentration). The concentrations of the natural polyamines and α-MeSpd were measured with the aid of high-pressure liquid chromatography as described previously (Hyvönen et al. 1992).

Immunoblotting

For immunoblotting, the skin samples were homogenized under keratin urea buffer [8 M urea, 50 mM Tris pH 7.6, 100 mM dithiothreitol, 0.13 M 2-mercaptoethanol, and 1tbl/50 ml complete protease inhibitor (Roche)]. Sample protein concentrations were determined using protein measurement kit (Bio-Rad Laboratories, Hercules, CA). Equal loadings of protein extract (10 µg) were separated in 10% dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under running buffer (25 mM Tris, 0.19 M glycine and 0.1% SDS). The transfer of separated proteins was carried out onto hydrophilic PVDF transfer membrane (Immobilon P, Millipore, Billerica, MA) using a semi-dry transfer system (Millipore). The transferred protein was fixed with methanol and the protein-loaded PVDF membrane was incubated with 5% of non-fat milk powder in 1× PBS buffer containing 0.1% Tween 20 (Sigma) for 24 h to block non-specific binding. Thereafter, the membranes were incubated with mouse polyclonal anti-actin (1:10,000) (Santa Cruz Biotechnology) and rabbit polyclonal anti-β-catenin (1:10,000) (Cell Signaling Technology, Boston, MA) for 1 h at room temperature. The membranes were washed five times each for 5 min with PBS buffer containing 0.1% Tween 20, and incubated for 40 min with 1:10,000 dilutions of goat anti-rabbit and anti-mouse IgG horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). The membranes were incubated with chemiluminescent substrate (Millipore), sealed into a plastic bag and placed into a film frame together with an autoradiography film (Kodak, Rochester, NY). The exposed film was developed with automatic developer (Kodak).

Statistical analyses

For statistical analyses, one-way ANOVA was used with Dunnett's post hoc test to compare the vehicle group to the spermidine-treated and α-MeSpd-treated groups.

Results

α-MeSpd activates hair growth in telogen phase mice

During anagen, the active growth of hair follicles and pigmentation occurs simultaneously in C57BL/6 mice (Muller-Rover et al. 2001). α-MeSpd treatment induced growth of hair in the telogenic C57BL/6 mice after 2 weeks of analog application. Hair growth was noticeable as a black spot at the application site (Fig. 1). Neither visible hair growth nor black coloration was seen in vehicle-treated (Fig. 1) or spermidine-treated groups (data not shown). Histology of the skin revealed active growth of hair follicles at the treatment site. However, in both controls and animals treated with natural spermidine, the hair follicles were confined to the proximal parts of epidermis, emphasizing its dormancy (Fig. 2).

α-MeSpd activates proliferation of cells residing in the hair follicle

Proliferating cell nuclear antigen (PCNA) is a marker antigen known to be upregulated during cell division (Maga and Hubscher 2003). We used PCNA to show the active proliferation of cells residing in the hair follicles. PCNA was upregulated in animals treated with α-MeSpd when compared with the vehicle group and spermidine group (Fig. 2). This result was verified by bromouridine (BrdU) staining (data not shown). Furthermore, cytokeratin 6, the hyperproliferation marker of keratinocytes, was expressed in the hair follicles of the animals treated with α-MeSpd, but not in animals treated with vehicle or natural spermidine. The treatment had no profound effect on the

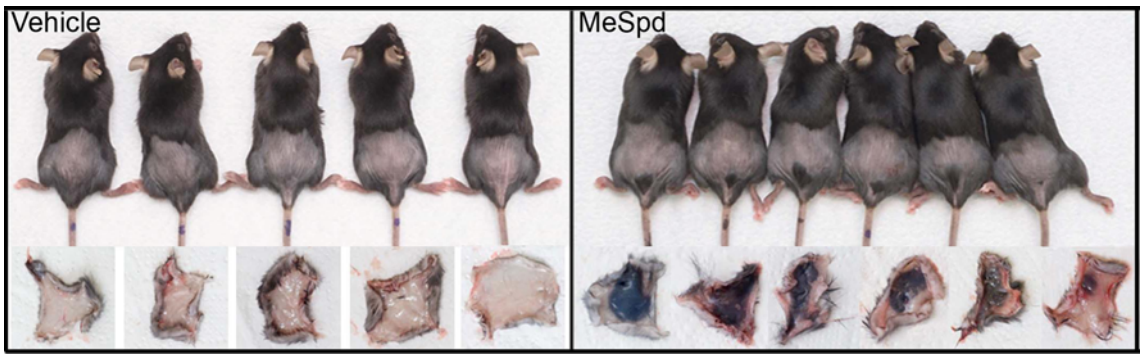

Fig. 1 Cutaneous application of α-MeSpd induces hair growth. The absence of hair growth was evident in shaved area in the control telogenic mice treated with the vehicle, whereas α-MeSpd treatment induced hair growth at the application site. The reverse sides of resected skins showed *dark gray* areas indicating anagen induction

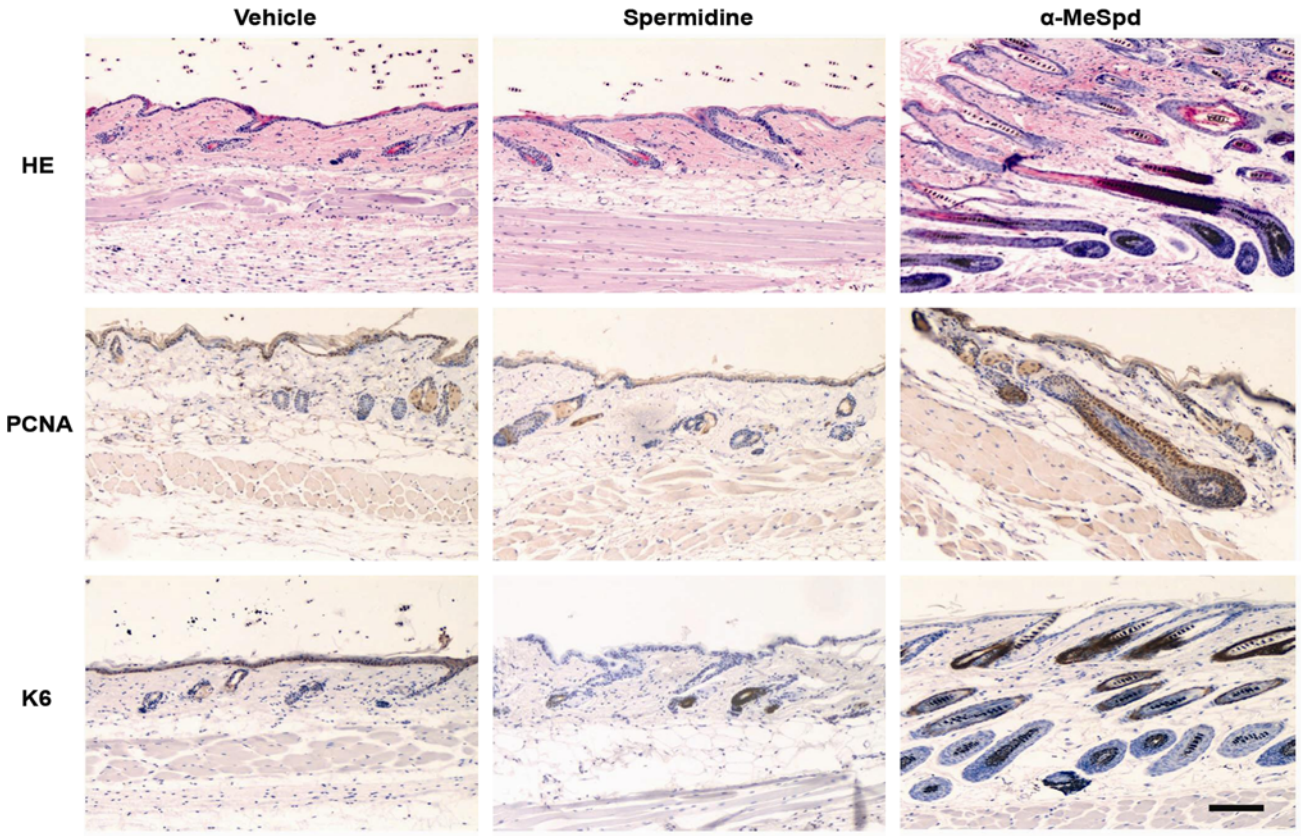

Fig. 2 α-MeSpd induces the growth of hair follicle. The effect of α-MeSpd treatment on the hair follicles of telogen mouse was analyzed with hematoxylin–eosin staining (*top row*). PCNA (*second row*) and cytokeratin 6 (K6) (*third row*) stainings showed increased proliferation of follicular cells, which is characteristic for anagen follicles. Vehicle and spermidine groups served as control. *Scale bar* = 100 μm

proliferation of the interfollicular keratinocytes which could be seen as a lack of cytokeratin 6 staining in interfollicular epidermis (Fig. 2). Neither did α-MeSpd have effect on the proliferation of cultured mouse primary keratinocytes. Furthermore, the analog was not able to rescue cultured cells from proliferation blockage initiated by calcium-mediated differentiation as described by Hennings et al. (1980) (results not shown).

α-MeSpd treatment upregulates the expression of β-catenin

β-Catenin is a signalling protein that plays a key role in normal hair development and cycling. Its expression is reported to be sufficient to activate the growth of hair follicle (Celso et al. 2004). Immunoblotting showed upregulation of β-catenin expression in the skin of α-

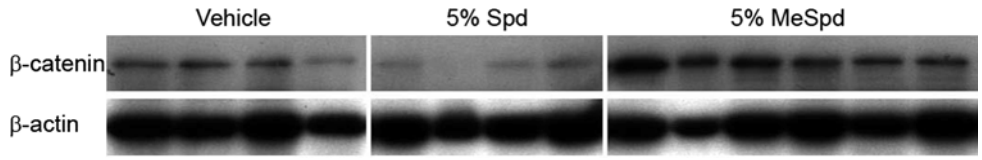

Fig. 3 α-MeSpd activates expression of *β*-catenin. The immunoblot depicts the expression level of *β*-catenin in animals treated with α-MeSpd and natural spermidine. The actin staining serves as a loading control

MeSpd-treated animals when compared with the vehicle-treated control group and animals treated with natural spermidine (Fig. 3).

α-MeSpd replaces natural spermidine

α-MeSpd has been shown to fulfill many of the functional roles of spermidine in rodent physiology (Järvinen et al. 2005). Table 1 illustrates the decreased concentration of natural polyamines in the epidermis of α-MeSpd-treated animals compared with the concentration of polyamines in the animals treated with natural spermidine or vehicle. Most notably, the concentration of spermidine was significantly decreased in epidermis ($P < 0.05$). However, the total pool of spermidine including the analog was significantly elevated in dermis. Also, cultured mouse keratinocytes incorporated α-MeSpd effectively, as more than half of the cellular spermidine was replaced by the analog after 4-day treatment (results not shown).

Discussion

The cells of anagen phase hair follicles are among the most rapidly dividing cells in the mammalian body (Vanscott et al. 1963). Dermal papilla is believed to be the source of factors that trigger the proliferation of cells, which ultimately evokes the onset of anagen. For example, growth factors such as keratinocyte growth factors, insulin-like growth factors and neuroendocrine peptides have demonstrated to maintain hair growth (Cotsarelis 1997). However, what actually regulates the expression of growth factors or other proliferation marker molecules during physiological hair growth phases has not yet been fully comprehended (Alonso and Fuchs 2006).

In our recent study, the polyamine pool in mouse skin was shown to increase during the anagen phase, and decrease during catagen and telogen (Pietilä et al. 2001). Disruption of this regulation leads to the cutaneous abnormalities seen in transgenic animals with disturbed polyamine metabolism. For example, the overexpressed polyamine biosynthesis in the outer root sheath keratinocytes leads to continuous proliferation of the keratinocytes and to displacement of functional follicles with large keratin-filled cysts (Soler et al. 1996). Accelerated polyamine biosynthesis makes these mice also prone to the cutaneous tumors (Megosh et al. 1995). On the other hand, the susceptibility to chemical carcinogenesis in skin can be lowered by inducing polyamine catabolism (Pietilä et al. 2001) or by overexpressing antizyme, an inhibitory protein of polyamine biosynthesis (Feith et al. 2007). The aforementioned cutaneous abnormalities can be cured and normal hair follicle function restored by inhibiting the overexpressed polyamine biosynthesis by cutaneous DFMO treatment (Soler et al. 1996). Interestingly, DFMO is also used for the treatment of hirsutism under the trade name of Vaniqa; the rationale being to inhibit the polyamine biosynthesis to the extent that the hair growth is halted (Shapiro and Lui 2001). We have now induced the hair growth artificially by elevating the polyamine pool of the mouse skin during telogen. The hair growth was induced even when the treatment reduced the epidermal concentrations of natural spermidine showing the capability of α-MeSpd to fully replace the physiological functions of its natural counterpart in the hair follicle function.

Long hair follicles that traverse the whole dermis seen at the application site are characteristic for anagen phase. PCNA upregulation in the skin of animals treated by α-MeSpd indicates that the growth of hair follicle was indeed attributed to the proliferation of keratinocytes. Polyamines have been shown to play indispensable in the progression of S-phase in cell cycle. This observation was strengthened by strong correlation between hepatic spermidine level and PCNA labeling index during liver regeneration (Alhonen et al. 2002).

Cytokeratin 6 is expressed in hair follicle keratinocytes undergoing proliferation and in interfollicular keratinocytes that are hyper-proliferative due to wounding, irritation or neoplastic growth (Freedberg et al. 2001). The confinement of cytokeratin 6 expression to the hair follicles and lack of hyper-proliferation in interfollicular epidermis indicate that α-MeSpd does not induce keratinocyte proliferation directly at cellular level. This view was strengthened by the unresponsiveness of cultured primary keratinocytes to the analog.

The hair growth induced by α-MeSpd was coupled with black pigmentation of skin at the application site. The black pigmentation underlines the follicular melanogenesis, which

Table 1 The effect of topical 5% α-MeSpd (w/v) treatment on the polyamine pools of epidermis and dermis

Tissue/treatment	Putrescine	Spermidine	Spermine	α-MeSpd	Total "Spermidine"
Epidermis					
Vehicle[a]	174 ± 71	145 ± 19	81 ± 20		145 ± 20
5% Spd	153 ± 50	240 ± 100	72 ± 23		239 ± 102
5% α-MeSpd	128 ± 38	102 ± 42*	51 ± 23	148 ± 34	250 ± 74
Dermis					
Vehicle[a]	201 ± 41	110 ± 29	43 ± 19		110 ± 29
5% Spd	196 ± 76	130 ± 28	41 ± 14		130 ± 28
5% α-MeSpd	227 ± 70	118 ± 49	44 ± 17	144 ± 55	263 ± 102**

Values are expressed as pmol/mg tissue wet weight

The vehicle and 5% Spd (w/v) groups served as controls. The last column shows the sum of the natural spermidine and α-MeSpd

Means ± SD

$n = 6$ ([a]$n = 5$). *$P < .05$; **$P < .01$

occurs simultaneously with anagen phase of the hair cycle (Slominski et al. 2005). During anagen phase, follicular melanocytes undergo a series of melanogenic activities. The transfer of melanin granules to medullary and cortical keratinocytes actively provides dark colorization to the shaft of the newly formed anagen hair (Slominski et al. 2005). Furthermore, many studies demonstrate simultaneous occurrence of follicular melanogenesis and anagen that is activated through application of exogenous hair growth promoting agents. For example, anagen activation by application of PDGF (Tomita et al. 2006) or the GPIGS peptide (Tsuruda et al. 2005) induced dark coloration to the newly growing hair and skin of mice. In agreement with these studies, the simultaneous follicular melanogenesis and hair follicle growth at the α-MeSpd application site suggests that the analog functions as a growth triggering agent for the whole hair follicle including the follicular melanocytes.

Hair follicle morphogenesis and growth are regulated by Wnt/β-catenin/Lef-1 signaling pathway (Millar 2002). Experimental data demonstrates the failure of the hair follicle growth by skin-specific depletion of β-catenin (Huelsken et al. 2001) or Lef-1 (van Genderen et al. 1994) or by overexpression of Wnt inhibitor (Andl et al. 2002). On the other hand, the growth of interfollicular epithelium is stimulated by constitutively activated overexpression of β-catenin (Gat et al. 1998). Furthermore, transient activation of β-catenin changed the telogen hair into the anagen hair at the site of exposure (Van Mater et al. 2003). During intestinal epithelial cell migration, polyamines have shown to regulate β-catenin phosphorylation and its free pool in cytosol (Guo et al. 2002). Polyamines are also shown to be involved in murine kidney development by controlling expression of c-ret, E-cadherin, and Pax2/8, all of which have implicated to have functions in epithelial–mesenchymal interactions (Loikkanen et al. 2005). The upregulation of β-catenin at the application site of α-MeSpd implies that the activation of hair follicles is due to normal epithelial–mesenchymal interaction.

The regulation of hair cycle has been under intense investigation, as it has been realized that the hair loss or unwanted hair growth largely reflect undesired changes in hair follicle cycling. The exact mechanism of hair cycle regulation is still largely unknown, but it is evident that hair cycle regulation is sensitive to changes in cellular polyamine content. As the cellular polyamine pool itself is finely tuned by extremely short living biosynthetic (Shantz and Levin 2007) and catalytic enzymes (Seiler 2004), it is likely that the polyamines serve as effector molecules in the hair cycle regulation. The inhibition of polyamine biosynthesis with DFMO is already used to treat unwanted hair growth (Shapiro and Lui 2001). Using an opposite approach, the present paper demonstrates for the first time that metabolically stable polyamine analogs may be useful in the treatment of hair loss.

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