Disturbed Keratinocyte Differentiation in Transgenic Mice and Organotypic Keratinocyte Cultures as a Result of Spermidine/Spermine N^1-Acetyltransferase Overexpression

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Overexpression of the rate-limiting enzyme in polyamine catabolism spermidine/spermine N^1-acetyltransferase (SSAT) in transgenic (Tg) mouse leads to accumulation of putrescine in the skin and permanent hair loss at the age of 3 wk. The hair follicles of these mice are replaced by dermal cysts and epidermal utriculi. Increased putrescine production is also seen in hyperproliferative cutaneous disorders such as in psoriasis. These disorders are characterized by delayed onset of epidermal differentiation characterized as reduced expression of terminal differentiation markers such as cytokeratins 1/10, and filaggrin and persisting expression of basal cell cytokeratins 5/14 in the suprabasal layers. The use of these markers in immunohistological analysis of SSAT Tg skin clearly showed signs of disturbed differentiation. To exclude the possibility that changes in differentiation originated from underlying connective tissue, we introduced SSAT gene into an established rat epidermal cell line. Organotypic cultures derived from the transfected cells displayed similar changes in their differentiation pattern as keratinocytes in Tg skin. The role of accumulated putrescine in cutaneous changes of SSAT Tg mice was verified by an experiment in which putrescine level was reduced by systemic putrescine biosynthesis inhibition. The putrescine reduction was sufficient to alleviate the cutaneous changes to such an extent that distinct hair regrowth could be seen. These results suggest that the cutaneous changes of SSAT Tg animals are due to disorders of the keratinocyte differentiation. Moreover, they strengthen the view that the proper regulation of polyamine metabolism plays an important role in the keratinocyte maturation.

Key words: cytokeratin/difluoromethylornithine/filaggrin/polyamines/putrescine
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The polyamines spermidine and spermine and their precursor putrescine are closely associated with growth and differentiation of mammalian cells. The exact mechanisms of their function are still largely unknown, although polyamines have been implicated in many physiological functions including DNA replication, transcription, translation, post-translational protein modification, and membrane stability. Some of these effects are specific for polyamines, whereas others are less specific due to the general cationic nature of these compounds. Irrespective of the specificity of their effects, polyamines are indispensable cellular components because their depletion, either by gene disruption or inhibitors of their biosynthesis, results in severe defects in cell growth (Cohen, 1998).

In attempts to elucidate the physiological roles of the polyamines, we have generated a number of transgenic (Tg) mouse and rat lines with genetically altered polyamine metabolism (Jänne *et al*, 2004). Activation of polyamine catabolism in mice by overexpressing spermidine/spermine N^1-acetyltransferase (SSAT) either under its own promoter (line UKU 165b) (Pietilä *et al*, 1997) or under mouse metallothionein I promoter (line UKU 181) (Suppola *et al*, 1999) is accompanied by thickening of the epidermis and hair loss. The hair follicles of these mice are replaced by dermal cysts and epidermal utriculi (pseudocomedones). Analysis of the skin polyamines revealed that the UKU 165b mice continuously overaccumulated putrescine, whereas spermidine content decreased and spermine content remained relatively unaltered compared with the levels of syngenic animals (Pietilä *et al*, 2001). Interestingly, identical hairless phenotype has been described for Tg mice overexpressing the key enzyme of polyamine biosynthesis, ornithine decarboxylase (ODC), under the cytokeratin 6 promoter (Peralta Soler *et al*, 1996). Paradoxically, both the activation of polyamine biosynthesis (overexpression of ODC) and that of polyamine catabolism (overexpresion of SSAT) led to similar hairless phenotype. Both Tg lines, however, show a massive overaccumulation of putrescine in the skin.

It has been suggested that an activation of polyamine biosynthesis is a necessary component of skin tumorigenesis (Koza *et al*, 1991). For example, induced ODC activity

Abbreviations: DFMO, α-difluoromethylornitine; EDTA, ethylenediamine tetraacetic acid; EGF, epidermal growth factor; ODC, ornithine decarboxylase; PCNA, proliferating cell nuclear antigen; REK, rat epidermal keratinocyte; SSAT, spermidine/spermine N^1-acetyltransferase; Tg, transgenic

and enhanced putrescine accumulation are essential for tumor promotion by 12-*O*-tetradecanoylphorbol-13-acetate (Pegg, 1986). The role of putrescine in neoplastic growth was clearly demonstrated by the use of a selective inhibitor of ODC (α-difluromethylornitine (DFMO)), which mainly affected the concentration of putrescine, blocked the formation of 7,12–dimethylbenz[*a*]anthracene, and initiated squamous papillomas in ODC Tg mice (Peralta Soler *et al*, 1998). Enhanced polyamine biosynthesis has also been shown to be associated with hyperproliferative states, like psoriasis (Lowe *et al*, 1982). In rare X-linked syndrome called keratosis follicularis spinulosa decalvans, characterized by follicular hyperkeratosis, a region on X chromosome containing SSAT gene is duplicated. Analyses of dermal fibroblasts of an affected individual indeed showed signs of activated polyamine catabolism such as three times increased SSAT activity, enhanced putrescine accumulation, and a decrease in cellular spermidine pool (Gimelli *et al*, 2002). Based on these results, proper regulation of putrescine pool either by biosynthesis or by polyamine turnover seems to have a pivotal role in epidermal neogenesis and proliferation.

ODC expression in chemically induced cutaneous papillomas in mice is associated with altered expression of keratins, which is seen as reduced expression of terminal differentiation markers such as cytokeratins 1 (K1)/10 (K10), and suprabasal expression of basal cell markers cytokeratins 5 (K5)/14 (K14) (Sundberg *et al*, 1994). The same altered differentiation pattern was also seen in the SSAT Tg mouse skin. To investigate the role of overaccumulated putrescine to the phenotype, we blocked its biosynthesis with systemic DFMO treatment. The treatment reduced the epidermal putrescine level to less than half from the original, which was sufficient to alleviate the epidermal differentiation defect. Interestingly, the treatment also reactivated the hair follicles to such an extent that distinct hair regrowth could be seen. We also introduced SSAT gene into a continuous rat epidermal cell line (rat epidermal keratinocyte, REK). The REK cell line forms a morphologically well-organized epidermis in the absence of feeder cells when grown on a collagen gel in culture inserts at an air–liquid interface (MacCallum and Lillie, 1990; Tammi *et al*, 2000). This unique organotypic culture model provides an easily maintained and reproducible model for studies of epidermal differentiation. The organotypic cultures prepared from the transfected SSAT overexpressing cells showed changes in their differentiation typical to the SSAT Tg skin. These results indicate that the interfollicular phenotype of SSAT Tg mouse skin is due to overaccumulation of putrescine, which leads to disturbed keratinocyte differentiation. Moreover, the results from the cell culture model suggest that the differentiation defect is intrinsic to the keratinocytes and does not originate from underlying basement membrane or connective tissue.

Results

Histological analysis of the SSAT Tg mouse skin The immunohistochemical staining (Fig 1*A*) showed that K14 expression in Tg skin is not confined to the basal layer as in wild-type (Wt) skin. Constant K14 expression was seen from the basal layer up to the cornified layer. Prominent expression was also seen inside the comedo-forming hair canals, which are distinctive to SSAT Tg mice (Pietilä *et al*, 2001). Both the terminal differentiation markers K1/10 and filaggrin were downregulated, which was seen as more diffuse immunostaining in Tg than in Wt skin. The proliferating cell nuclear antigen (PCNA) staining from multiple sections from three different animals of both Wt and Tg gave statically different mean numbers of proliferative cells in 39 ± 7 and 104 ± 9 per mm of interfollicular epidermis ($p < 0.001$), respectively. This increase on PCNA positive cells was partly due to suprabasally proliferative cell. Extensive PCNA staining was also seen in the basal cells surrounding the epidermal comedones and in the dermal cysts. Contrary to the PCNA expression, the marker of keratinocyte hyperproliferation, K6, was not present in the interfollicular epidermis of the Tg skin.

By acetylating higher polyamines, SSAT facilitates their catabolism to putrescine and promotes their excretion out of cells (Casero and Pegg, 1993). This reduction of polyamines is compensated by activation of polyamine biosynthesis. Thus, the massive accumulation of putrescine in various tissues of SSAT mouse is most likely due to both enhanced polyamine turnover by SSAT and compensatory elevated biosynthesis. To address the question of whether the putrescine accumulation is directly responsible for the abnormalities of the SSAT Tg skin, we blocked its biosynthesis with DFMO, which is a highly specific irreversible inhibitor for the putrescine biosynthesis by ODC. This compound has been previously used to reactivate the hair growth of Tg mice overexpressing ODC under K6 promoter (Peralta Soler *et al*, 1996).

After 2 wk of 0.5% (wt/vol) DFMO administration, the epidermal putrescine in SSAT Tg skin was reduced to less than half from the original level whereas spermidine and spermine levels remained relatively unaltered (Table I). The effect of putrescine reduction was immunohistochemically seen as a clear downregulation of epidermal K 14 expression (Fig 1*B*). Based on the K14 staining, the follicular cysts of Tg mice are, presumably, derived from outer root sheath keratinocytes. Interestingly, as a first indication of follicle reactivation, K14 negative cells appeared in the middle of the dermal cysts in DFMO-treated animals (Fig 1*B*). Eventually, the cysts started to elongate and formed functional hair follicles. After 4 wk of treatment, the treated SSAT Tg mice showed newly formed hair (Fig 2).

Generation of REK cells overexpressing SSAT and assessment of their differentiation in organotypic cultures Since SSAT enzyme is normally expressed in very low amounts, little is known about the tissue specificity of SSAT expression. It is likely, however, that the SSAT transgene driven by it own promoter is expressed in every cell type of the skin. Based on the metallothionein expression in the skin (Karasawa *et al*, 1991), the metallothionein promoter in the mouse line UKU 181 most likely directs the expression of SSAT to proliferating keratinocytes in the hair follicle and to the basal layer of the epidermis. Since the cutaneous phenotypes of these mouse lines are indistinguishable, it seems that the keratinocytes are the most susceptible cell

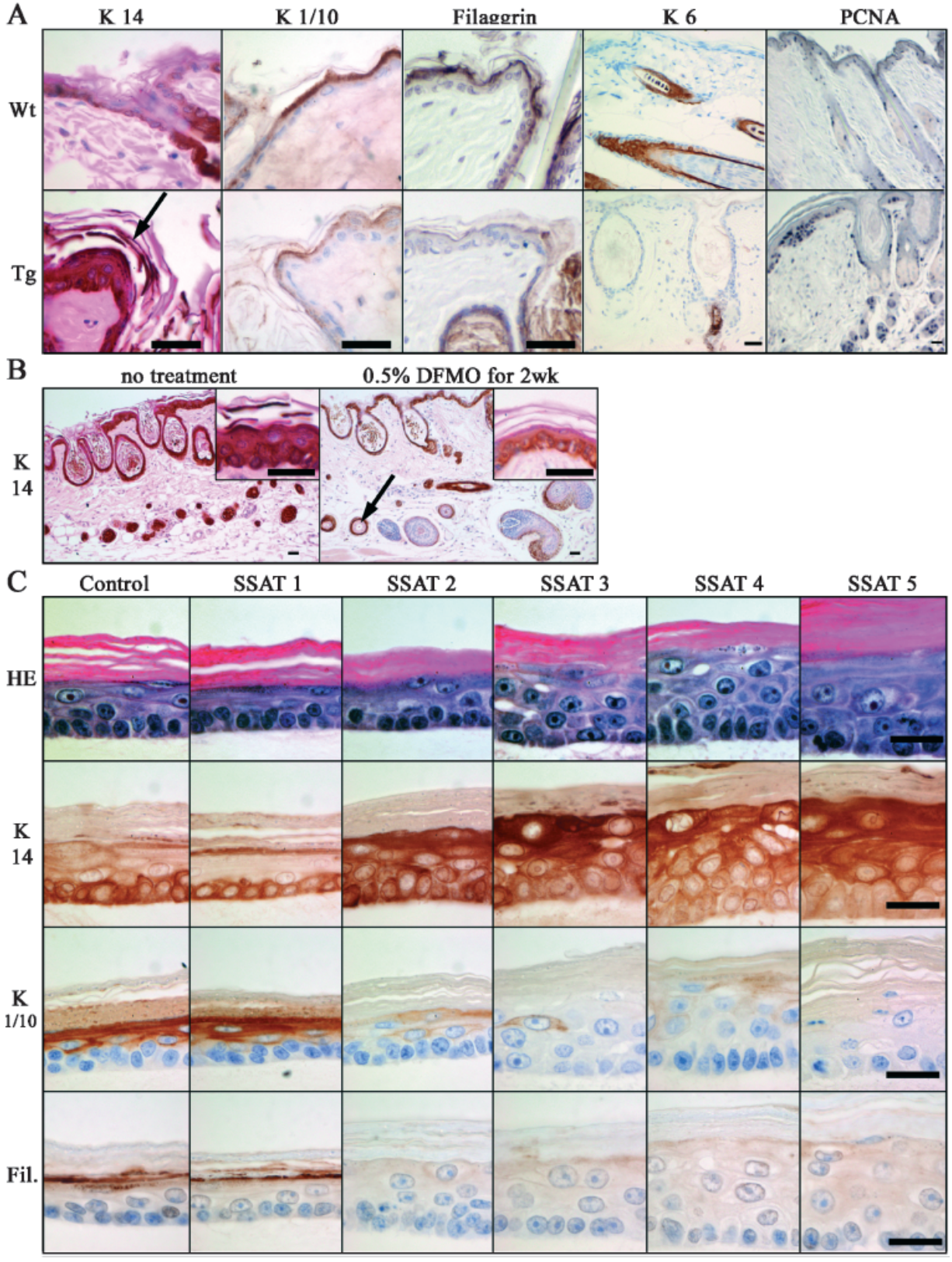

Figure 1
Localization of K14, K1/10, filaggrin, K6, and proliferating cell nuclear antigen (PCNA) in mouse skin and organotypic cultures. (*A*) Skin sections of wild-type (Wt) and spermidine/spermine N^1-acetyltransferase (SSAT) transgenic (Tg) mice were immunostained with antibodies against cytokeratins 14, 1/10, filaggrin, cytokeratin 6, and PCNA, as indicated. Note the abundant immunostaining of K14 in cornified layer in Tg skin and the downregulation of K1/10 and filaggrin. Regardless of a high number of PCNA positive cells, no K6 expression can be seen at the interfollicular epidermis of Tg mice. (*B*) The effect of 0.5% α-difluromethylornitine (DFMO) on K 14 expression and hair follicle activation after 2 wk treatment. Note the normalization of epidermis seen as downregulation of K14 (greater magnification in the upper right corner) and the appearance of K14-negative cells in the middle of the dermal cysts. (*C*) Hematoxylin–eosin and immunostained sections of organotypic cultures derived from SSAT Tg clones (SSAT 1–5) and a control clone transfected only with selection marker. Note the persisting immunostaining of K14 form the basal cell to more superficial layers and the clearance of K10 and filaggrin in the clones SSAT 2–5. *Scale bar* = 25 μm.

Table I. Effect of polyamine synthesis inhibition on the epidermal polyamine pools

Animal + treatment	nmol per mg protein		
	Putrescine	Spermidine	Spermine
Wt	5.4 ± 3.5	38.5 ± 4.6	9.1 ± 2.4
Wt + DFMO	4.0 ± 1.3	33.2 ± 4.1	9.9 ± 2.6
Tg	52.8 ± 7.5	25.7 ± 4.3	8.9 ± 0.6
Tg + DFMO	23.4 ± 2.2***	25.1 ± 1.7	10.8 ± 1.1

The mice were treated with 0.5% DFMO in their drinking water for 2 wk.
Means ± SD. n = 5.
***p < 0.001.
Wt, wild-type; DFMO, α-difluoromethylornitine; Tg, transgenic.

type to SSAT overexpression. To address whether the changes due to SSAT overexpression are intrinsic to the keratinocytes, we introduced the SSAT transgene to REK cells.

Five co-transfected clones were selected according to their high SSAT activity (Table II). Both the original REK cells and two selected control clones, containing only the neomycin resistance construct, formed a well-organized epidermis where basal cells expressed K14 and suprabasal layers expressed differentiation markers K1/10 and filaggrin (only one of the controls is shown) (Fig 1*C*). Also, keratohyalin granules and the cornification of keratinocytes, both indicative of epidermal differentiation, were clearly visible. The SSAT Tg clone (SSAT 1) with only moderately enhanced SSAT activity had morphology and immunohistochemical staining pattern similar to the control clone. The other four SSAT Tg organotypic cultures (SSAT 2–5) had more elevated SSAT activities and disorganized structures, since vital cells could be seen at the more superficial layers compared with the control culture. As in the skin of the SSAT-mouse, the K14 expression was not confined to the basal layer and the reduction of K1/10 and filaggrin expression was even more prominent than in the skin of the SSAT Tg animals. Similar to SSAT mouse epidermis, the REK organotypic cultures did not express any K6 (not shown). Since both the control and SSAT Tg REK cells proliferate with an extremely high rate, no correlation between SSAT expression and PCNA staining (not shown) could be made. Determination of the polyamine concentrations revealed an enhanced accumulation of putrescine in SSAT Tg clones compared with the control clones (Table II). Unfortunately, there is no correlation between putrescine levels and the differentiation in the SSAT Tg clones, as the clone SSAT 2 has less putrescine than clone SSAT 1. The low putrescine level in clone SSAT 2 may be due to the significantly reduced spermidine, which attenuates the putrescine accumulation by polyamine turnover. Correspondingly, due to reduced spermine and spermidine amounts the clone SSAT 5 have similar amount of putrescine as SSAT 4 despite the higher SSAT activity.

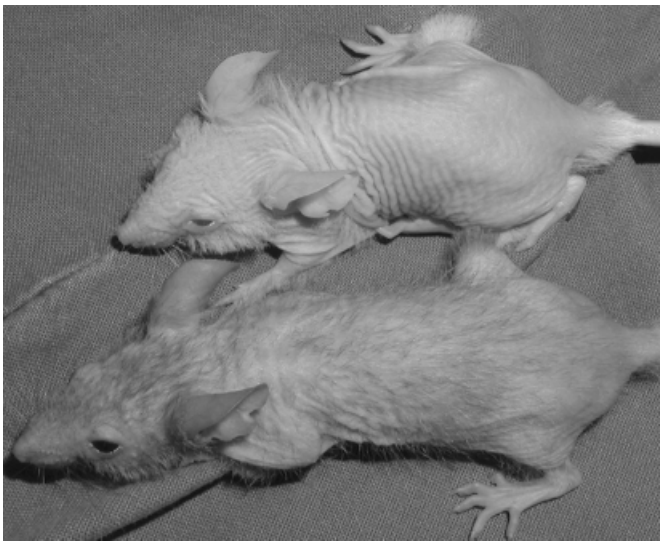

Figure 2
Reduction of putrescine reactivates the hair growth. Two spermidine/spermine N^1-acetyltransferase transgenic littermates are presented. The mouse in front has been treated with 0.5% (wt/vol) α-difluromethylornitine in drinking water for 4 wk whereas the mouse in the back belonged to the control group receiving plain tap water. Note the newly formed hair on the back of the treated animal.

Table II. SSAT activities and polyamine levels in the SSAT transgenic REK clones (SSAT 1–5) and a control clone transfected only with selection marker

	SSAT activity (pmol per mg protein per min)	nmol per mg protein		
		Putrescine	Spermidine	Spermine
Control	3.13 ± 1.49	1.31 ± 1.14	34.6 ± 8.64	17.1 ± 4.17
SSAT 1	15.4 ± 1.64***	8.33 ± 0.74***	34.5 ± 2.17	13.8 ± 1.72
SSAT 2	37.9 ± 10.9**	4.63 ± 1.08*	18.7 ± 3.75*	9.90 ± 1.89
SSAT 3	19.4 ± 1.51***	12.5 ± 1.19***	31.1 ± 1.14	14.1 ± 0.87
SSAT 4	44.6 ± 4.59***	24.0 ± 1.64***	27.4 ± 1.33	14.9 ± 0.87
SSAT 5	79.4 ± 16.2**	24.0 ± 3.81***	20.5 ± 1.94	8.35 ± 1.13*

The REK cells were cultivated in monolayers to sub-confluence and harvested.
Means ± SD. n = 3.
*p < 0.05,
**p < 0.01,
***p < 0.001.
SSAT, spermidine/spermine N^1-acetyltransferase; REK, rat epidermal keratinocytes.

Discussion

We have previously shown that the hair loss and other cutaneous abnormalities in the SSAT Tg mouse are in all likelihood due to overaccumulated putrescine (Pietilä *et al*, 2001). In this study, we have investigated further the underlying mechanisms of the cutaneous changes by using various proliferation and differentiation markers. An abnormal expression pattern, where K14 was not confined to the basal layer, was immunohistochemically shown both in the SSAT Tg skin and the SSAT-overexpressing organotypic cell culture, hence demonstrating that the Tg keratinocytes maintained undifferentiated charasteristics up to the superficial layers. Hyperproliferation of the Tg epidermis was also apparent as indicated by the increased number of PCNA-positive cells—from which a great deal was due to suprabasaly proliferating cells. In various hyperproliferative skin diseases, the proliferation is accompanied by disappearance of differentiation markers, such as K 1/10 and filaggrin. This was also seen in the SSAT Tg skin and transfected cell cultures. As a result, for this disturbed differentiation the SSAT mice have a "rhino-like" appearance where the cornified layer of the epidermis becomes extremely thick upon aging. Presumably, this thickening is not only due to the enhanced proliferation but also due to the disturbed cornification process that prevents the normal scaling off of the keratinocytes.

The role of putrescine in cutaneous abnormalities of SSAT mouse was clearly demonstrated when systemic DFMO treatment alleviated the epidermal differentiation defect and turned the dermal cyst to functional hair follicles. The hair follicle reactivation may not be only due to normalization of keratinocyte differentiation as hair follicle function has been shown to be finely tuned by a variety of local mediators. The dermal cysts of SSAT mouse, however, originate from disintegrated hair channels (Pietilä *et al*, 2001) and they seem to harbor basal keratinocytes from thee outer root sheath based on the K14 expression. The basal cells in the outer root sheath have been shown to originate from the same stem sell population as do the basal cells in epidermis (Morris, 2004 #187). Thus, it seems probable that putrescine has the same effect on the keratinocytes both in the epidermis and hair follicle. Indeed, the first indication of hair follicle reactivation in DFMO treatment was the normalization of keratinocyte differentiation manifested as the appearance of K14-negative cells in the middle of the cysts. The proliferation of cells in follicular cysts seems to be dependent on putrescine concentration as doubly Tg mice overexpressing, both ODC and SSAT and with extremely high levels of putrescine in the skin, showed distinctly larger cysts compared with mice overexpressing only SSAT (Pietilä *et al*, 2001). Based on present and previous results, it seems that a high putrescine concentration maintains the basal cell phenotype of keratinocyte, both in hair follicle and epidermis, by promoting the proliferation and by prohibiting the differentiation. Putrescine may also have a role in maintaining at least some of the cells in dermal cyst in stem cell state as these cysts could be reformed to functional hair follicles by DFMO treatment.

Both in normal and diseased skin, keratinocyte proliferation and differentiation are controlled by growth factors, such as epidermal growth factor (EGF) and transforming growth factor beta (Hashimoto, 2000). EGF has been shown to induce both the biosynthesis (Fitzpatrick *et al*, 1987) and the uptake of polyamines into the cell (Milovic *et al*, 1995). Furthermore, depletion of the polyamines has shown to upregulate the expression of EGF receptor (Manni *et al*, 2001). Interestingly, EGF has a similar hyperproliferative effect on REK cells (Pasonen-Seppänen *et al*, 2003) as we have shown in this study by overexpressing SSAT. Thus, there is a possibility that EGF and other local mediators affect keratinocyte proliferation and differentiation by stimulating the synthesis of putrescine.

The direct mechanism by which putrescine prohibits keratinocyte differentiation is not clear. Putrescine as a small cationic molecule may compete with calcium (Ca^{2+}), which is essential for keratinocyte differentiation. Putrescine may also compete directly with isopeptide formation during cornification of keratinocytes as it is a natural substrate for transglutaminases (Piacentini *et al*, 1988) and thus prevent the differentiation process.

Several factors that have been shown to regulate keratinocyte differentiation have also been connected to the regulation of the polyamine metabolism: glucocorticoids (Russell *et al*, 1978), retionic acid (Zheng *et al*, 1995), and EGF (Fitzpatrick *et al*, 1987). It has been difficult, however, to evaluate whether the polyamines have some intrinsic role in differentiation or whether the polyamine pool changes are just a result of some process in differentiation. In this article, we have presented an animal and a cell culture model that demonstrate the feasibility of intervening the polyamine pools by overexpression of SSAT. Moreover, the results show that it is possible to regulate differentiation of keratinocytes by changing their polyamine content.

Materials and Methods

Tg animals The SSAT Tg animals used in this study were members of line UKU 165b (Pietilä *et al*, 1997). Non-Tg littermates served as controls. DFMO (ILEX Oncology, San Antonio, Texas) treatment was started after weaning (4–5-wk-old) and it was given in the drinking water *ad libitum* at a concentration of 0.5% (w/v). The study protocols were approved by the Animal Care and Use Committee of the University of Kuopio, Finland.

Cell culture A keratinocyte cell line derived from newborn rat skin (REK) (Baden and Kubilus, 1983) was used for the transfection and the organotypic culture. The stock cultures were grown in minimum essential medium (Gibco, Invitrogen, Carlsbad, California) with 10% fetal bovine serum (HyClone, Logan, Utah), 4 mM L-glutamine (Sigma, St Louis, Missouri), 50 μg per mL streptomycin sulfate, and 50 U per mL penicillin (Sigma) at 37°C in humidified 95% air/5% CO_2. For selection, 0.5 mg per mL G418 (Gibco, Invitrogen) was added to the medium. REK were passaged twice a week at a 1:5 split ratio using 0.05% trypsin (wt/vol) (Gibco, Invitrogen), 0.02% ethylenediamine tetraacetic acid (EDTA) (wt/vol) (Sigma) in phosphate-buffered saline (PBS). The cells were co-transfected with pGT-N28 plasmid with aminoglykoside phosphotransferase as a selectable marker (New England Biolabs, Beverly, Massachusetts) and pUC-19 (New England Biolabs) plasmid with the SSAT transgene. The transfection was carried out by using LipofectAMINE PLUS (Gibco, Invitrogen) reagent. The transgene construct contained all the exons and introns of the SSAT gene together with 3 kbp of the 5′-flanking region and 11.5 kbp of the 3′-flanking region. The control cells were transfected only with the selection plasmid.

For organotypic cultures, REK were cultured at the air–liquid interface on type I collagen support as described in Pasonen-Seppänen *et al* (2001).

Histology and Immunohistochemistry The organotypic cultures and the skin samples were fixed in Histochoice (Amresco, Solon, Ohio) overnight, washed with 0.1 M sodium phosphate buffer (pH 7.4), dehydrated in graded ethanol, embedded in paraffin, and cut into 4 μm sections for standard hematoxylin–eosin staining or for immunohistochemistry. Rabbit polyclonal antibodies were used for filaggrin (PRB-417P), and K6 (PRB-169P) (Covance Research Products, Berkeley, California) detection. Guinea-pig polyclonal antibody was used for K14 (GP CK 14.2, Research Diagnostics, Flanders, New Jersey) detection. Mouse monoclonal antibodies were used for PCNA (PC-10, Santa Cruz Biotechnology, Santa Cruz, California), and K1 and K10 (LH1, BioDesing, Saco, Maine) detection. For visualization of bound antibodies, the following imunohistochemical kits were used according to the manufacturer's protocols: for rabbit polyclonal antibodies, Poly-HRP anti-Rabbit IgG IHC kit (ImmunoVision Technologies, Brisbane, California), for guinea-pig polyclonal antibody Vectastain ABC kit (Vector Laboratories, Burlingame, California), and for mouse monoclonal Poly-HRP anti-mouse/rabbit IgG IHC kit (ImmunoVision Technologies).

SSAT activity and polyamines The dermis of mouse skin was scraped off with a scalpel and the epidermis was homogenized for polyamine concentration determination. The SSAT enzyme activity was determined from REK culture homogenates in 25 mM Tris-HCl pH (7.4), 1 mM dithiothreitol, 0.1 mM EDTA, and 0.1% Triton X-100 as described previously (Libby, 1978). Polyamine concentrations were measured with the aid of HPLC (Hyvönen *et al*, 1992).

Statistical analyses Students's unpaired two-tailed *t* test was used for statistical analyses.

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