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Relation of Skin Polyamines to the Hairless Phenotype in Transgenic Mice Overexpressing Spermidine/Spermine N^1-Acetyltransferase

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We recently generated a transgenic mouse line with activated polyamine catabolism due to overexpression of spermidine/spermine N^1-acetyltransferase. Phenotypic changes in these animals included permanent loss of hair at the age of 3 wk. We have now further explored development of hair loss during early postnatal life. The first hair cycle appeared to be completed normally in the transgenic animals. At postnatal day 15, although macroscopically indistinguishable from their syngenic littermates, the transgenic animals already showed microscopically signs of hair follicle degeneration. Wild-type mice started their second anagen phase at day 27, whereas the transgenic animals did not display functional hair follicles at that time. Hair follicles were replaced by dermal cysts and epidermal utriculi. Analysis of skin polyamines revealed that the transgenic animals continuously overaccumulated putrescine. The view that an overaccumulation of putrescine was related to the disturbed hair follicle development was strengthened by the finding that doubly transgenic mice overexpressing, both spermidine/spermine N^1-acetyltransferase and ornithine decarboxylase and with extremely high levels of putrescine in the skin, showed distinctly more severe skin changes compared with the singly transgenic animals. Interestingly, in spite of their hairless phenotype, the spermidine/spermine N^1-acetyltransferase transgenic mice, were significantly more resistant to the development of papillomas in response to the two-stage skin carcinogenesis. Analysis of skin polyamines indicated that the syngenic mice tripled their spermidine content when exposed to promotion, whereas the transgenic animals showed only modest changes. These results suggest that putrescine plays a pivotal part in normal hair follicle development. ***Key words: dermal cyst/hair cycle/ornithine decarboxylase/putrescine/skin tumorigenesis. J Invest Dermatol 116:801–805, 2001***

We recently described two transgenic mouse lines overexpressing spermidine/spermine N^1-acetyltransferase (SSAT) under its own promoter (Pietilä *et al*, 1997) or governed by mouse metallothionein (MT) I promoter (Suppola *et al*, 1999). These animals showed all signs of activated polyamine catabolism in their tissues, namely the appearance of N^1-acetylspermidine, not normally found in mouse tissues, striking overaccumulation of tissue putrescine, and decreases in spermine and/or spermine pools. In addition to the changes of tissue polyamines, the transgenic animals displayed phenotypic changes not readily explainable by the distorted tissue polyamine pools. These included permanent loss of hair due to the replacement of normal hair follicles by large dermal cysts and epidermal utriculi (pseudocomedones), female infertility, and an apparent lack of subcutaneous fat deposits (Pietilä *et al*, 1997). Interestingly, identical hairless phenotype to SSAT mice, as regards the onset of hair loss and histologic changes in the skin, has recently been described for transgenic mice overexpressing ornithine decarboxylase (ODC) under the control of keratin promoter (Soler *et al*, 1996). It may appear paradoxical that both the activation of polyamine biosynthesis (overexpression of ODC) as well as enhanced polyamine catabolism (overexpression of SSAT) leads to the same hairless phenotype. Both transgenic lines share a common feature, however, namely a massive overaccumulation of putrescine in the skin. Thus, excessive accumulation of putrescine could contribute to the observed dermal abnormalities. It has been suggested that an activation of polyamine biosynthesis is a necessary component of skin tumorigenesis (Koza *et al*, 1991). Indeed, enhanced papilloma formation was detected in an earlier study where transgenic mice overexpressing ODC were exposed to two-stage skin tumorigenesis, where 7,12-dimethylbenz[*a*]anthracene (DMBA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were used for initiation and promotion, respectively (Halmekytö *et al*, 1992). Moreover, mice expressing the ODC under the control of keratin 6 promoter developed papillomas directly after initiation by DMBA without a subsequent promotion with TPA (O'Brien *et al*, 1997). In line with the latter animals, inhibitors of ODC, such as α-difluoromethylornithine, have been used to prevent papilloma formation during two-stage skin carcinogenesis (Takigawa *et al*, 1982, 1983; Weeks *et al*, 1982). The lack of hair is not an

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Abbreviations: DMBA, 7,12-dimethylbenz[*a*]anthracene; MT, metallothionein;ODC, ornithine decarboxylase; SSAT, spermidine/spermine N^1-acetyltransferase; TPA, 12–*O*-tetradecanoylphorbol-13-acetate.

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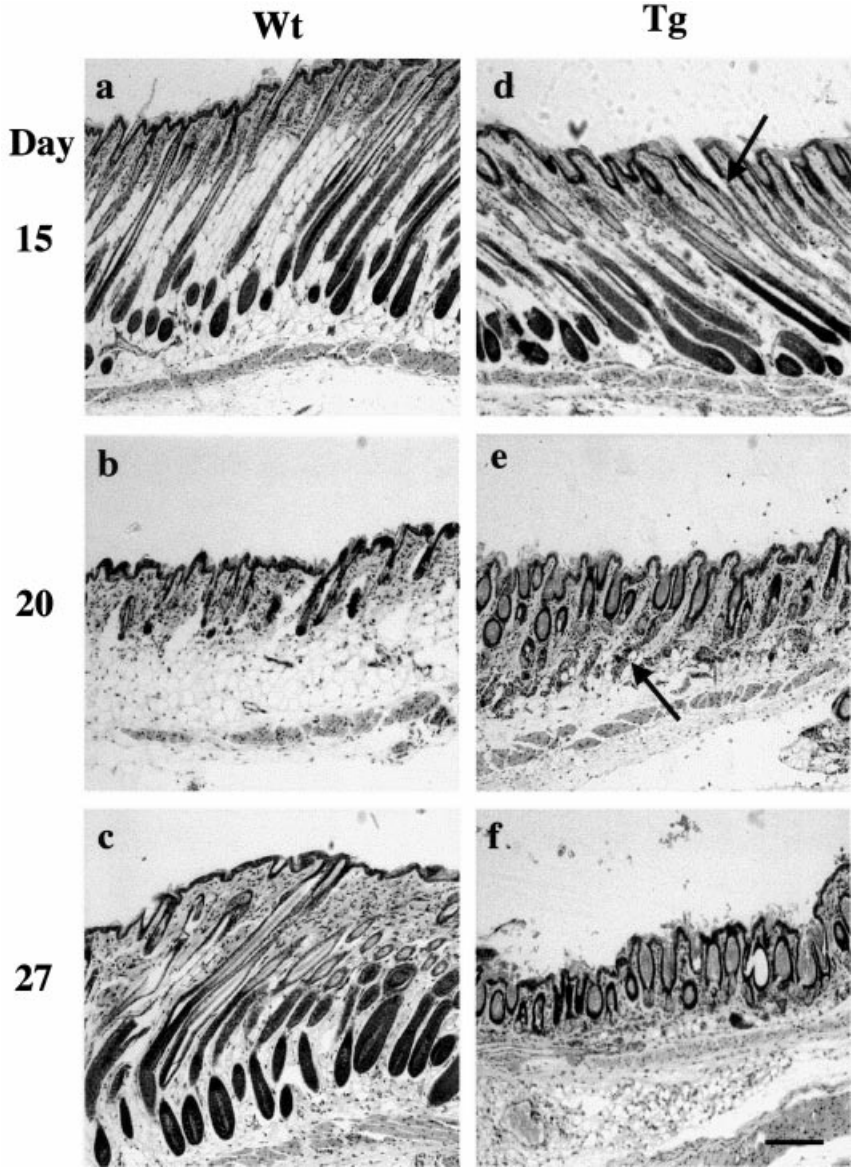

Figure 1. Dermal changes around the time of first catagen. Skin sections of mice before the first catagen (15th postnatal day) at the top, during the first telogen (20th postnatal day) at the middle and at the time of second anagen (27th postnatal day) at the bottom. The sections at the right side are from SSAT transgenic (Tg) and at the left are the sections from their wild-type (Wt) siblings. Note the widened hair canals in (*d*) and the disintegrated remnants of epithelial strand in (*e*). No functional hair follicles or thickening subdermal fat layer can be seen in (*f*) at the time of second anagen. *Scale bar*: 200 μm.

uncommon feature among different mouse strains. Hairless phenotype can be resulted, for example, from a single gene mutation, such as in the ''rhino'' (hr^{rh}) strain (Howard, 1940). The hairless (*hr*) gene encodes a putative zinc-finger transcription factor, which is expressed in the hair follicle, epidermis, and brain (Cachon-Gonzalez *et al*, 1994). Interestingly, the histologic changes of SSAT mouse skin are almost identical to those described for the rhino mouse. These changes include the appearance of keratin filled utriculi, the loss of subcutaneous fat and complete lack of skin tone. Also the life span of SSAT animals is greatly shortened, as it is with the rhino mice (Davies *et al*, 1971). In this study we have further explored the SSAT overexpressing mice with regard to the histologic changes associated with the onset of hair loss and their relation to the polyamine pools in the skin during early postnatal development. It appears to us that putrescine has a distinct role in the murine hair cycle. Somewhat unexpected was the finding that these hairless animals seemed to be significantly more resistant to the two-stage skin tumorigenesis than their syngenic littermates.

MATERIALS AND METHODS

Transgenic animals The transgenic animals used in this study were members of the lines UKU 48 (MT-ODC) (Alhonen *et al*, 1996), UKU 165b (SSAT) (Pietilä *et al*, 1997) and UKU 181 (MT-SSAT) (Suppola *et al*, 1999). Nontransgenic littermates served as controls.

Early postnatal development For the examination of postnatal development of the skin, seven different littermate groups were studied at the age of 0, 5, 10, 15, 20, 27, and 41 d. The mice were killed by cervical dislocation and whole (epidermis + dermis) skin samples were taken for histologic and biochemical analyses. Transgenicity was determined by polymerase chain reaction as described previously (Pietilä *et al*, 1997).

Simultaneous overexpression of SSAT and ODC Hybrids of ODC and SSAT overexpressing mice lines were used to force the putrescine production further both by enhanced synthesis of putrescine from ornithine and accelerated catabolism from spermidine. Both the transgenes were expressed under mouse MT I promoter instead of their natural promoters to avoid transcriptional regulation by distorted polyamine pools. Nonhybrid siblings were grouped, with the aid of polymerase chain reaction, to wild-type, MT-ODC and MT-SSAT animals. The mice were killed at the age of 4 mo by cervical dislocation and whole skin samples were taken for histologic and biochemical analysis.

Two-stage skin carcinogenesis Twelve week old female transgenic and syngenic animals were studied. The skin tumorigenesis was initiated by a topical application of 200 nmol of DMBA in acetone on to dorsal skin. Two weeks after the initiation, TPA (10 nmol in acetone) was

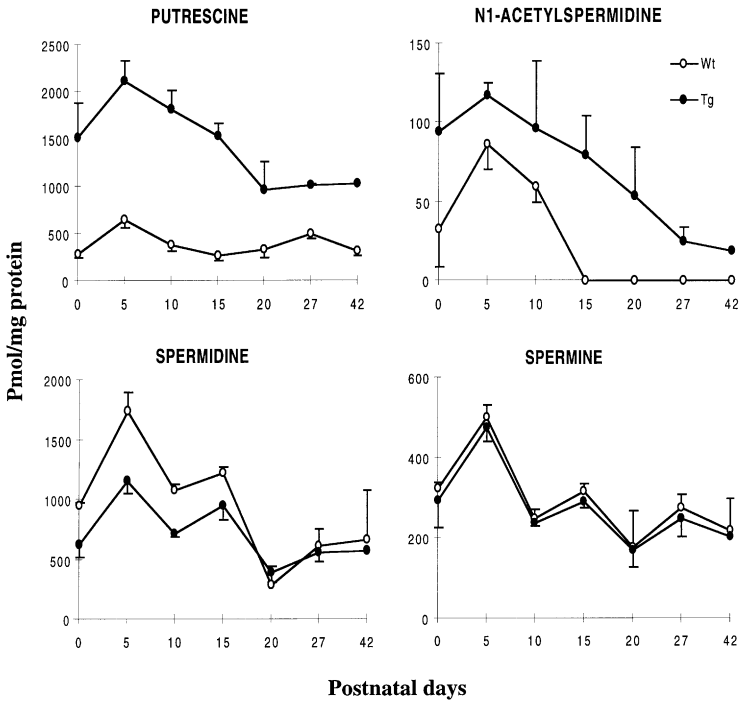

Figure 2. Polyamine levels in skin samples from different age groups. Animals were killed at seven different time points in their early postnatal life, and whole skin samples were taken for polyamine determinations. Wt, wild type; Tg, transgenic. The values are mean ± SD.

applied twice a week (Gonzalez and Byus, 1991). Sixteen weeks after the initiation, the mice were killed by cervical dislocation and the papillomas were counted and histologically examined. Both syngenic and transgenic animals were divided into four groups (10 animals in each) as follows: untreated controls, DMBA treatment followed by twice-weekly applications of acetone, initiation with acetone followed by twice-weekly promotion with TPA and initiation with DMBA followed by twice-weekly promotion with TPA.

Analytical methods The enzyme activities and polyamine concentrations were determined from the homogenized whole skin samples. Polyamine concentrations were measured with the aid of high-performance liquid chromatography (Hyvönen *et al*, 1992). The activity of ODC (Jänne *et al*, 1991) and SSAT (Libby, 1978) were determined as described previously. The protein concentration was determined as described in Bradford (1976).

Histologic analysis The skin samples were fixed either in buffered formalin or paraformaldehyde, dehydrated through ethanol, embedded in paraffin and 5 μm sections were cut for standard hematoxylin–eosin staining or for apoptosis detection (TUNEL) by using In Situ Cell Death Detection Kit, Fluorescein (Boehringer Mannheim, Mannheim, Germany).

Statistical analyses Student's unpaired two-tailed t test or analysis of variance when applicable was used for statistical analyses. $p < 0.05$ was considered significant.

RESULTS

Early postnatal development The mouse hair growth is cyclic and is composed of anagen (hair growth), catagen (follicle regression), and telogen (resting) phases. Macroscopically, the SSAT mice lost their hair at the age of 3 wk, but microscopic signs of hair loss were seen already 1 wk earlier. Newborn wild-type and transgenic mice were indistinguishable from each other and the first hair cycle appeared to be identical in syngenic and transgenic pups. Microscopically, first changes related to hair loss in the transgenic animals were found at postnatal day 15, when both strains still were macroscopically identical. The earliest sign was a dilatation of the hair follicle at its most distal portion adjacent to the epidermis (**Fig 1*d***). At this point some of the hair follicles of both wild-type and transgenic were already in catagen. This could be clearly seen from the TUNEL-labeled sections (not shown), where clear apoptotic cells had appeared around the dermal papilla in the regressing hair matrix; however, no apparent differences in the amount or the distribution of apoptotic cells between the mice groups were seen. Five days later, after the first catagen, superficial utriculi formation was clearly seen (**Fig 1*e***). Similar remnants of hair follicle disintegration, which have been described in the skin of hairless mouse (Panteleyev *et al*, 1999), can also be seen at this stage. The wild-type mice had already started their second anagen at the 27th postnatal day, which could be seen as extended hair follicles in thickened hypodermal fat layer (**Fig 1*c***), whereas the transgenic mice did not have any functional hair follicles and the hypodermal fat layer remained thin (**Fig 1*f***). The cyclicity of the hair growth appeared to be accompanied by similar changes in the levels of spermidine and spermine both in the wild-type and transgenic animals (**Fig 2**). Elevated polyamine levels are supposed to be associated with cell proliferation and decreased levels with cell death or cell rest (Jänne *et al*, 1977). As depicted in **Fig 2**, putrescine overaccumulation in the skin of newborn SSAT mice was striking. The putrescine level of transgenic skin also stayed at elevated levels throughout postnatal development and adulthood. In addition to elevated putrescine pools in the transgenic skin, also the content of N^1-acetylspermidine was higher and sustained longer than in the nontransgenic skin (**Fig 2**).

Simultaneous overexpression of SSAT and ODC In order to force putrescine overaccumulation further, we generated a doubly transgenic mouse line overexpressing both SSAT and ODC under the control of mouse MT promoter. The putrescine levels in the skin of hybrid mice overexpressing both ODC and SSAT were significantly elevated in comparison with mice overexpressing either ODC or SSAT alone (**Table I**). The levels of the higher polyamines, however, were similar in the skin of syngenic and transgenic mice (**Table I**). As shown in **Fig 3**, the dermal abnormalities, which included large dermal cysts and epidermal utriculi, were more pronounced in the hybrid animals. Area quantitation of dermal cysts measured from multiple sections from three different animals of both MT-SSAT and hybrid MT-ODC × MT-SSAT lines gave statically different mean areas of 6.7×10^3 μm^2 and 13.6×10^3 μm^2 ($p < 0.019$), respectively. The differences of epidermal utriculi between the mice groups were not as obvious and no statically significant differences were

Table I. Effect of ODC, SSAT, and ODC × SSAT overexpression under MT promoter in mouse skin enzyme activities and pools[a]

	Enzyme activity		Polyamine pools (pmol per mg protein)			
Animals	ODC (pmol per h per mg)	SSAT (pmol per min per mg)	Putrescine	N^1-acetylspermidine	Spermidine	Spermine
Wild type	76 ± 66	13.5 ± 1.5	505 ± 85	0	1030 ± 70	450 ± 155
MT-ODC	1003 ± 228	14.2 ± 3.7	635 ± 65	0	1330 ± 560	520 ± 110
MT-SSAT	105 ± 41	85.2 ± 9.5	1950 ± 125	310 ± 60	1330 ± 280	425 ± 50
MT-ODC × MT-SSAT	6009 ± 650***	127 ± 39.2	2840 ± 360***	230 ± 30	1170 ± 125	315 ± 110

[a]The whole skin samples were taken from 4 mo old siblings. Data are mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (refers to the statistical differences between MT-SSAT and MT-ODC × MT-SSAT groups).

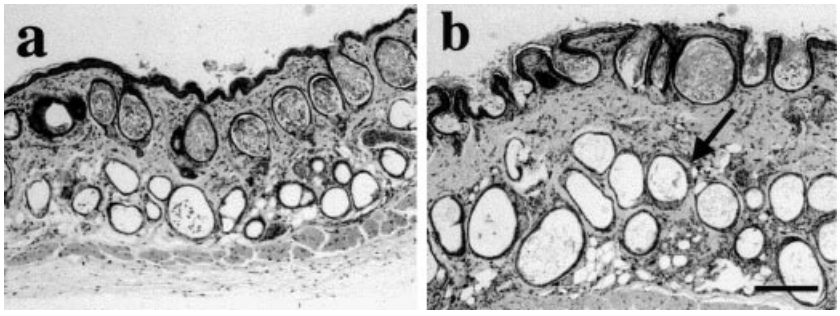

Figure 3. The relationship of putrescine levels to dermal abnormalities. The skin sections have been taken from 4 mo old MT-SSAT (*a*) and MT-ODC × MT-SSAT double transgenic (*b*) siblings. Note the enlarged dermal cysts and the corresponding thickening of the dermis. *Scale bar*: 200 μm.

obtained, as the mean areas of MT-SSAT and MT-ODC × MT-SSAT lines were 13.4×10^3 μm^2 and 15.2×10^3 μm^2, respectively.

Two-stage skin carcinogenesis In chemical two-stage skin carcinogenesis only the mice receiving both the initiation (DMBA) and promotion (TPA) developed papillomas (**Table II**). The SSAT mice were significantly more resistant to the treatment as the number of papillomas per animal on dorsal skin of the SSAT *vs* syngenic mice were 2.8 ± 2.3 and 10.5 ± 9.2, respectively. As regards the skin polyamine pools, both TPA and the combination of DMBA and TPA approximately tripled the spermidine level in wild-type animals, whereas the response in the transgenic animals in this respect was significantly less pronounced (**Table II**). Interestingly, TPA and the combination strikingly stimulated ODC activity in the wild-type mice, whereas the treatments were without effect in the transgenic mice (**Table II**).

DISCUSSION

In this study it appears that SSAT overexpressing mice developed normal hair at the time of first anagen, but severe abnormalities became evident during the first hair follicle regression (catagen), resulting in complete loss of hair. There are similarities between the events leading to hair fall in SSAT and rhino mice lines, which include the widening of distal hair canals and disintegration of epithelial strands at the time of first catagen. Neither functional hair follicles nor thick subdermal fat layer associated with anagen were seen after the first catagen. Upon aging of the SSAT mice, the dermal cysts and epidermal utriculi became larger, leading to extreme wrinkling of the skin. The dermal cysts of SSAT mice seemed to be derived from the remnants of epithelial strands as also found in rhino mutant mice (Panteleyev *et al*, 1999). The epidermal utriculi, also common to rhino mouse, were keratin-filled pseudocomedones apparently derived from blocked hair canals. Striking induction of polyamine synthesis is associated with hair growth at anagen (Sundberg *et al*, 1994). It has been hypothesized that local mediators induce ODC transiently to trigger the anagen phase of hair follicle growth. When concentrations of local mediators decline, the ODC activity declines and hair follicles enter the catagen phase. Both the mice overexpressing the ODC under keratin 6 promoter (Soler *et al*, 1996) and mice overexpressing SSAT have constitutively high levels of intracellular putrescine. This constitutive accumulation of putrescine seems to favor continuous proliferation of epithelial cells possibly leading to the formation of follicular cysts. This abnormal proliferation could also be seen as occasional thickening of the epidermis. The possible role of putrescine as a potential regulator of hair growth was further strengthened by the finding indicating that hybrid mice massively producing putrescine both by an enhanced synthesis through overexpression of ODC and activated catabolism of the polyamines through overexpression of SSAT not only had significantly higher skin putrescine pools but also displayed more severe skin histopathology than animals only overexpressing SSAT. At least one group has suggested, that SSAT induction can lead to cytotoxicity and apoptosis by hydrogen peroxide (based on a cell line study with polyamine analogs), which is produced by polyamine oxidase catalyzed degradation of acetylated polyamines (Ha *et al*, 1997). Neither premature nor enhanced apoptosis, however, was seen in TUNEL-labeled sections of SSAT mouse. Moreover, we have recently shown that fibroblasts isolated from fetuses of SSAT transgenic mice were extremely sensitive to polyamine analogs, yet an inhibition of polyamine oxidase did not attenuate the anti-proliferative effect of SSAT induction (Alhonen *et al*, 1998). It is, therefore, unlikely that the dermal abnormalities are due to increased hydrogen peroxide production by enhanced polyamine turnover. As to the mechanisms of skin abnormalities the possibility remains that extreme tissue pools of putrescine somehow interfere DNA-binding activity of zinc-finger proteins, such as the hairless gene product. In fact, putrescine has been shown to inhibit the binding of estrogen receptors to its cognate response element (Lu *et al*, 1998). Rhino mice are extremely sensitive to chemical tumorigenesis and ultraviolet radiation-induced tumor development (Davies *et al*, 1971). Interestingly, transgenic mice overexpressing (ODC) under the control of keratin

Table II. Effect of two-stage skin tumorigenesis treatment to polyamine activities and pools in the skin of SSAT mice (Tg) and their wild-type (Wt) littermates[a]

Treatment		No. of papillomas/animal	Enzyme activity		Polyamine pools (pmol per mg protein)			
			ODC (pmol per h per mg)	SSAT (pmol per min per mg)	Putrescine	N^{1}-acetylspermidine	Spermidine	Spermine
None	Wt	0	76 ± 66	13.5 ± 1.5	507 ± 85	0 ± 0	1030 ± 70	450 ± 155
	Tg	0	381 ± 43**	114.2 ± 13.2***	2390 ± 240***	250 ± 30***	1010 ± 220	420 ± 130
TPA	Wt	0	720 ± 330	27.3 ± 9.2	1160 ± 340	110 ± 100	3040 ± 920	780 ± 300
	Tg	0	361 ± 91**	98.1 ± 17.4	4330 ± 570***	320 ± 50***	1860 ± 220***	590 ± 110
DMBA+TPA	Wt	10.5 ± 9.2	546 ± 190	21.9 ± 7.3	800 ± 200	160 ± 80	2790 ± 360	520 ± 110
	Tg	2.8 ± 2.3*	276 ± 56***	87.1 ± 8.8	3870 ± 400***	280 ± 50***	1700 ± 220***	410 ± 80*

[a]The skin tumorigenesis was initiated by a topical application of 200 nmol of DMBA in acetone onto dorsal skin. Two weeks after the initiation, TPA (10 nmol in acetone) was applied twice a week for 14 wk. The mice were killed and samples were taken from the application area for analysis. Data are mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (refers to the statistical differences between wild-type and transgenic groups).

6 promoter are also sensitized to chemical tumorigenesis (Soler *et al*, 1996; O'Brien *et al*, 1997). It has been suggested that the activation of polyamine biosynthesis is a necessary component of skin tumorigenesis (Koza *et al*, 1991). This view is supported by a number of studies where inhibitors of ODC, such as α-difluoromethylornithine, have been employed to prevent polyamine biosynthesis and papilloma formation during the two-stage skin tumorigenesis (Takigawa *et al*, 1982, 1983; Weeks *et al*, 1982). In spite of their almost identical hairless phenotype, the SSAT mice thus distinctly differ from the ODC overexpressing and the rhino mice by being significantly more resistant to chemical tumorigenesis than their nontransgenic littermates. Whether this resistance is related to the inability of TPA to induce ODC and/or to expand skin spermidine pools in the SSAT overexpressing mice remains to be elucidated.

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