Modulation of Murine Hair Follicle Function by Alterations in Ornithine Decarboxylase Activity

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Mice that overexpress a mutated ornithine decarboxylase (ODC) transgene in outer root sheath keratinocytes of the hair follicle were used to study the role of this enzyme in regulating hair follicle structure and function. These transgenic mice have a normal first hair cycle, but lose their hair completely beginning 2–3 wk after birth. Transgene overexpression in follicular keratinocytes is first detected at day 12 after birth, coincident with the development of follicular cysts in the upper portion of the dermis. The onset of keratin 6 expression also begins around day 12; because the promoter/regulatory region of the bovine keratin 6 gene was used to target ODC transgene expression to hair follicle keratinocytes, these data demonstrate the faithful temporal and cell type–specific expression of the K6-driven transgene. The ODC inhibitor 2-difluoromethylornithine could prevent hair loss and partially normalize skin histology if administered before the onset of ODC overexpression. 2-Difluoromethylornithine could also reactivate hair growth in animals with complete hair loss. Our results suggest that ODC is an important regulatory gene for the mouse hair follicle. ***Key words: polyamines/2-difluoromethylornithine. J Invest Dermatol 106:1108–1113, 1996***

Hair growth in mice and other mammals, including humans, is characterized by a cyclic process of follicle regeneration/hair growth (anagen), follicle involution/hair growth cessation (catagen), and a follicle resting stage (telogen). The factors that regulate the mammalian hair cycle are largely unknown, although some promising candidates have emerged from studies on transgenic mice: fibroblast growth factor 5 (Hébert *et al*, 1994) and the transforming growth factor-β superfamily members BMP-2 and BMP-4 (Blessing *et al*, 1993). Whereas these growth factors and signaling molecules may be important humoral mediators of follicle growth and regression, what is completely lacking are clues to *intracellular* regulators of hair follicle activity. In an immunocytochemical analysis of ornithine decarboxylase (ODC) expression in murine epidermis *versus* epidermal tumors, Sundberg *et al* (1994) observed hair cycle–dependent ODC expression in follicles. In telogen follicles, ODC expression was upregulated in a small cluster of outer root sheath cells near the so-called "bulge" region, whereas in anagen follicles, expression was seen along most of the length of the follicle. In normal interfollicular epidermis, elevated levels of ODC are never observed unless the skin is treated with inducing stimuli such as phorbol esters (O'Brien *et al*, 1975a; Weeks *et al*, 1982).

In experimental models of skin carcinogenesis (largely mouse models), several lines of evidence suggest that non-melanoma tumors frequently arise from presumptive stem cells of the hair follicle (reviewed in Miller *et al*, 1993). Based on previous work in the two-stage model of skin carcinogenesis (O'Brien *et al*, 1975b; O'Brien, 1976), we hypothesized that constitutive overexpression of ODC might enhance susceptibility of epidermal keratinocytes to neoplastic transformation. We therefore decided to construct transgenic mice that constitutively overexpress ODC in hair follicle keratinocytes. The resulting transgenic mice, which overexpress ODC in outer root sheath keratinocytes, indeed exhibit a high frequency of spontaneous skin tumors, including keratoacanthomas, which are known to develop from hair follicles (Megosh *et al*, 1995). One of the other interesting phenotypes of these transgenic mice was hair loss beginning at 2–3 wk of age, when the second cycle of hair growth would be expected to begin. Histologic analysis of the skin of adult transgenic mice with complete hair loss revealed follicular cysts in the dermis that progressively increased in size as the animals aged (Megosh *et al*, 1995). Because ODC is an enzyme for which potent inhibitors are available, we decided to ask whether modulation of ODC transgene activity could influence hair follicle function in these mice. If so, these transgenic mice could be a useful experimental model to analyze intrinsic and extrinsic factors that regulate the mammalian hair follicle.

MATERIALS AND METHODS

Transgenic Mice The production of transgenic mice that overexpress a mutated ODC specifically in skin has been described in detail elsewhere (Megosh *et al*, 1995). For the experiments described herein, transgenic progeny from the founder line designated K6–55 were used. The ODC transgene is maintained in the hemizygous state by matings of transgenic with B6C3F1 mice. Typically, male hemizygous transgenic mice are mated with normal B6C3F1 female mice to generate approximately equal numbers of transgenic pups and nontransgenic littermates. When necessary, polymerase chain reaction analysis of tail DNA was used to identify transgenic mice, as described previously (Megosh *et al*, 1995). When 2-difluoromethylornithine (DFMO) was administered, it was dissolved in drinking water at a concentration of 1% (w/v).

Immunocytochemistry Skin for both routine histology and immunocytochemistry was fixed overnight in Fekete's solution (61% ethanol, 3.2% formaldehyde, 0.75 N acetic acid) and embedded in paraffin. Immunocytochemistry was performed on 5-μM sections using rabbit anti-ODC

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Abbreviations: DFMO, 2-difluoromethylornithine; K6, keratin 6.

Figure 1. Transgenic mice have multiple skin abnormalities. Shown are a transgenic mouse (*bottom*) and its normal littermate, derived from a B6C3F1X founder cross. Note excessive skin folds, wrinkling, and longer than normal nails in the transgenic mouse.

antiserum at 1:3,000 and a rabbit anti-mouse K6 antiserum at 1:8,000, with an avidin-biotin detection system (Vector Laboratories, Burlingame, CA).

ODC and Polyamine Analyses Skin specimens were separated into epidermal and dermal fractions by brief heat treatment (55°C, 20 s), followed by scraping with a razor blade. Tissues were homogenized in buffer (25 mM Tris-HCl, pH 7.5, containing 1 mM dithiothreitol and 0.1 mM ethylenediamine tetraacetic acid) or 0.2 N perchloric acid for ODC and polyamine analyses, respectively. Assays were conducted exactly as described previously (Koza *et al*, 1991). For ODC activity, 1 unit = 1 nmol CO_2 liberated/h at 37°C.

RESULTS

Transgenic Mice That Overexpress ODC Have Multiple Abnormalities Except for smaller size, transgenic mice of both sexes appeared phenotypically normal for approximately 2 wk after birth. They then exhibited progressive hair loss, including vibrissae, which was complete by 6 wk of age. Unless specifically treated (see below), alopecia was irreversible. Nail growth was also accelerated, and as the animals aged, the skin became increasingly wrinkled and folded because of the increase in size and number of dermal follicular cysts. Early in life, transgenic mice were smaller than normal littermates, but with age, transgenic animals achieved similar weights as normal mice. The weight distribution, however, was obviously very different; among mice of similar weight, the skin of normal mice represented 25% of total carcass weight, whereas in older transgenic mice, skin weight was 55% of total carcass weight because of the greater area and thickness of the skin (A. Peralta Soler and T. G. O'Brien, unpublished data). A transgenic mouse and a normal age-matched littermate are shown in **Fig 1**.

The phenotype of these mice resembles in some respects that of rhino mice, an allelic variant of the hairless (*hr/hr*) mouse (Mann, 1971). Although the integration site of the ODC transgene has not yet been mapped, it is very unlikely that it is at the *hr* locus on chromosome 14; offspring from six different founders all displayed the same phenotypic manifestations. Moreover, the ODC transgene acts in a dominant manner, whereas the *hr* mutation is recessive.

Morphologic Changes and Transgene Expression First Appear at About Day 12 After Birth In transgenic mice that overexpress ODC, the first hair growth phase was normal but subsequent cycles did not occur. To determine the timing of the onset of transgene expression relative to morphologic changes, we euthanized nontransgenic and transgenic pups at 2- to 3-d intervals from 2 to 23 d after birth and prepared skin sections for histology and immunocytochemistry. There was no obvious difference in skin histology between nontransgenic and transgenic littermates until day 12 postnatally, when small follicular cysts and moderate sebaceous cell hyperplasia were observed in transgenic skin **(Fig 2*b*)**. By day 23, the follicle-derived cysts were larger and more numerous, and normal follicles were absent **(Fig 2*d*)**. In terms of ODC expression, up to 10 d after birth there were no apparent differences in the pattern of ODC expression in transgenic *versus* nontransgenic skin as determined immunocytochemically (data not shown). At 12 d, however, there was elevated ODC expression in some developing cysts and follicular areas of the transgenic skin **(Fig 2*b*)** compared with controls **(Fig 2*a*)**. By 23 d, ODC overexpression in transgenic skin was clearly associated with developing pilar and follicular cysts **(Fig 2*d*)**, with weaker expression in follicles of normal skin **(Fig 2*c*)**.

The results of immunocytochemical analysis of ODC expression in newborn mice of different ages suggested that the first signs of follicular abnormalities were observed in the upper portion of the follicle (i.e., the infundibulum). As shown in **Fig 3**, at 16 d there was clear evidence of incipient cyst formation in the upper half of the follicle, whereas the lower half of the follicle, including the hair bulb and dermal papilla, appeared normal. Inspection of multiple sections of full-length follicles from 12- to 16-d old animals did not

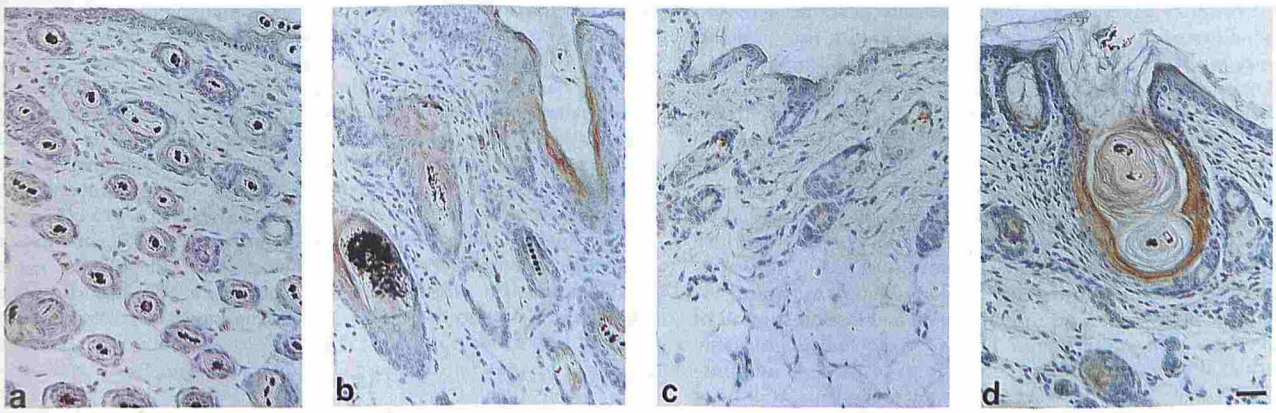

Figure 2. ODC is overexpressed in follicles and in incipient cysts of transgenic mice. Pups from a B6C3F1 x transgenic cross were euthanized at various days after birth, skin samples were taken for histologic and immunocytochemical analysis, and liver tissue was removed for polymerase chain reaction determination of transgene status. *a,c*, Sections from nontransgenic pups at 12 and 23 d after birth, respectively; *b,d*, transgenic pups at the same ages. *Scale bar*, 100 μm.

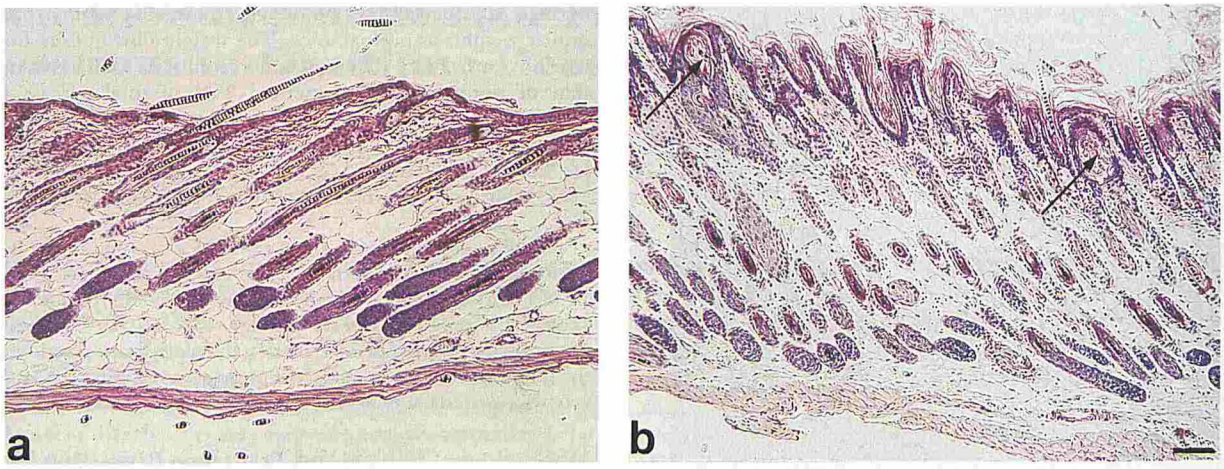

Figure 3. Abnormalities first develop in the upper portion of follicle. Histology is shown for normal (*a*) and transgenic (*b*) skin at 16 d. Note incipient cysts in the infundibular area (*arrows*) and normal-appearing hair bulb and dermal papilla in transgenic skin. *Scale bar*, 300 μm.

reveal any obvious abnormalities of the lower follicle (including the hair bulb) in transgenic mice.

If, as is likely, the more intense staining of ODC in transgenic skin at day 12 and thereafter is due to activation of transgene expression (as opposed to expression of the endogenous ODC gene), then there may be a temporal association between the onset of keratin 6 promoter-driven ODC expression and keratin 6 expression itself. This question was addressed using the same series of skin sections analyzed for ODC expression, except that a polyclonal anti-K6 antiserum was used instead. As shown in **Fig 4*a,b***, there was no evidence of K6 expression in either normal or transgenic skin at 4 d postnatally. There was also no histologic difference observed at this time. The first evidence of K6 expression was seen at day 12 in nontransgenic mice. Expression was restricted to the outer root sheath of anagen hair follicles in the mid-dermis **(Fig 4*c*)**; this is the predicted expression pattern based on the results of others (Heid *et al*, 1986; Ramírez *et al*, 1995). Interestingly, K6 staining of transgenic skin revealed a different expression pattern: Follicles on the mid-dermis were negative, but weak staining was detected in follicles of the upper dermis, some of which appeared to be incipient pilar cysts **(Fig 4*d*)**. This is very similar to the expression pattern for ODC at day 12 in transgenic mice **(Fig 2*d*)**. By day 23, virtually all hair follicles in nontransgenic mice were positive **(Fig 4*e*)**, whereas K6 expression in transgenic mice was generally restricted to follicular cysts **(Fig 4*f*)**.

DFMO Prevents Hair Loss To determine whether elevated transgene expression was directly responsible for the hair-loss phenotype, we used DFMO, the irreversible inhibitor of ODC. This compound has been used in mice previously and is both effective at inhibiting ODC and well tolerated at doses of up to 2% in the drinking water (Weeks *et al*, 1982; Takigawa *et al*, 1982; Kingsnorth *et al*, 1983). Newborn pups, both transgenic and nontransgenic, were administered DFMO at 1% (w/v) in their dam's drinking water. At weaning, transgenic mice (identified by polymerase chain reaction analysis of tail DNA) were divided into two groups: One group was switched to regular drinking water, and one group continued to receive drinking water containing 1% DFMO. Typically, weanling transgenic mice had lost most or all of their hair, but mice administered DFMO were indistinguishable from normal B6C3F2 mice. All transgenic mice (n = 4) administered DFMO from birth continued to maintain normal-appearing hair coats, whereas those mice switched to regular drinking water at week 3 gradually lost hair and developed the thickened, wrinkled skin typical of transgenic mice never exposed to DFMO **(Fig 5*a*)**. Histologically, skin from a transgenic mouse maintained continuously on DFMO had normal, hair-containing follicles **(Fig 5*b*)**, whereas skin from a mouse returned to regular drinking water had typical follicle-derived cysts **(Fig 5*c*)**. Follicle density in transgenic mice maintained continuously on DFMO was, however, less than that in nontransgenic skin of the same age.

Previous results had indicated that ODC transgene overexpression in skin occurs predominantly in the dermis, where follicular cysts are localized (Megosh *et al*, 1995). DFMO treatment was clearly effective in inhibiting ODC activity in transgenic dermis **(Table I)**. Withdrawal of DFMO led to a recovery of transgene expression, but not to levels typically observed in untreated transgenic dermis (Megosh *et al*, 1995; T.G. O'Brien, unpublished results). Putrescine levels reflected the relative ODC activities in transgenic skin (continuous DFMO and DFMO-withdrawn) and nontransgenic skin, whereas, curiously, spermidine and spermine levels did not; both were elevated in each of the transgenic treatment groups *versus* normal dermal levels of these polyamines (Megosh *et al*, 1995). The explanation for the elevations in spermidine and spermine levels in mice continuously administered a concentration of DFMO that completely inhibits ODC expression is not known. Thus, it appears that putrescine levels, and not spermidine or spermine, in the dermis correlate best with hair growth status: Normal (low) levels of putrescine are permissive for normal hair follicle activity, whereas high levels disrupt the hair cycle.

DFMO Reactivates Hair Growth To address the question of whether transgene inhibition in older animals with complete hair loss could reactivate hair growth, we administered DFMO (at 1%) in the drinking water to 6.5-wk-old transgenic mice with no normal hair follicles in the skin. After 2 wk of DFMO administration, hair growth in all treated mice (n = 3) had begun (compare **Fig 6*a* *versus* 6*b***), and by 9 wk of drug treatment, substantial hair growth had occurred (compare **Fig 6*c* *versus* 6*d***). Compared with control transgenic skin **(Fig 6*e*)**, DFMO-treated skin contained normal hair follicles, with fewer and smaller follicular cysts **(Fig 6*f*)**. As expected, ODC and polyamine levels were also normalized in DFMO-treated skin **(Table II)**. These results indicate that transgene expression sufficient to cause complete hair loss does not irreversibly impair the ability of follicle stem cells to generate normal hair follicles.

DISCUSSION

The transgenic mouse model we have described here and elsewhere (Megosh *et al*, 1995) implicates ODC as a gene that, when overexpressed, can disrupt hair follicle structure and function. We presume that the effects of ODC overexpression are mediated by the substantial elevations in putrescine, and to a lesser extent,

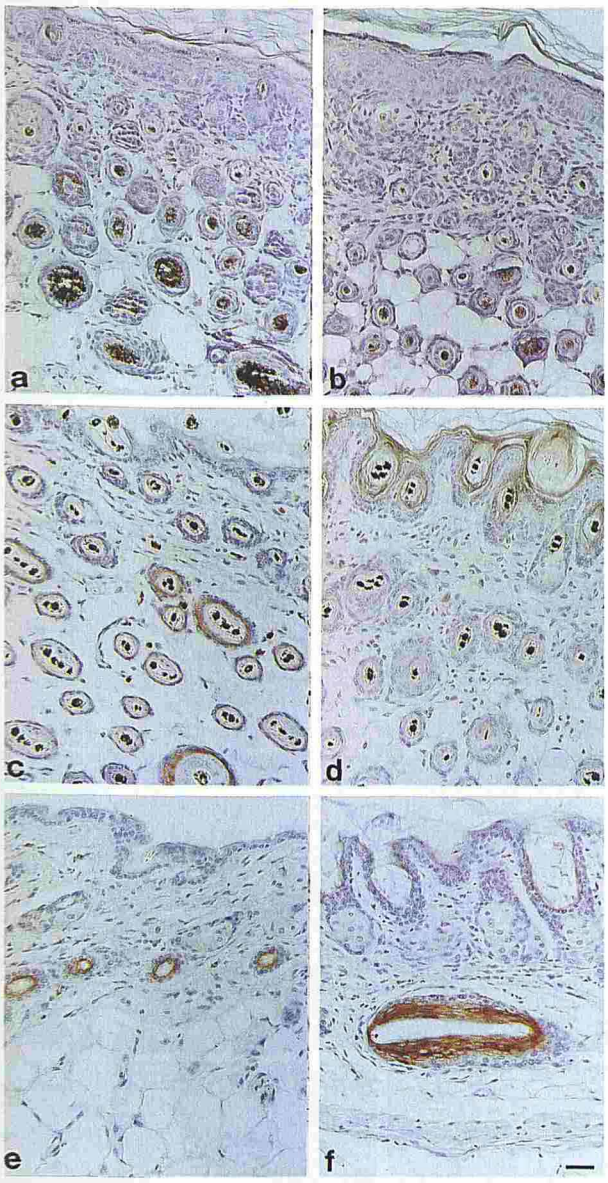

Figure 4. K6 expression is first observed on day 12 after birth. Results are shown for K6 immunocytochemistry of mice at 4 d (*a,b*), 12 d (*c,d*), and 23 d (*e,f*) after birth. *a,c,e*, Nontransgenic skin; *b,d,f*, transgenic skin. *Scale bar*, 100 μm.

spermidine, that are observed in the dermis of transgenic mice, because no functions other than its enzymatic activity have been rigorously ascribed to ODC. ODC is almost always a very low abundance protein in normal adult mammalian tissues, but Sundberg *et al* (1994) have reported high levels of ODC expression in anagen hair follicles, compared with more restricted expression in telogen follicles of adult CD-1 mice. This follicular stage–specific expression of ODC in a normal outbred mouse and our results in transgenic mice overexpressing ODC are consistent with the following hypothetical role for ODC (and polyamines) in follicle behavior. Local mediators (fibroblast growth factors, BMPs?) induce ODC in a transitory fashion to trigger the anagen phase of follicle growth. Enzyme activity (and follicle growth) is maintained by the continuous presence of mediator substances. When the local concentration of exogenous factor(s) declines, ODC is de-induced, and follicles enter the catagen phase. If ODC activity is constitutively expressed at a very high level (as in transgenic mice), the resulting high levels of intracellular polyamines suppress normal follicular behavior and instead favor continuous proliferation of outer root sheath keratinocytes to form follicular cysts. At least some cells in these cysts, however, retain stem cell capability once ODC and polyamine levels are reduced to normal values (e.g., via DFMO administration).

The bovine K6 promoter used to drive ODC transgene expression in this mouse model appears to direct ODC expression to the "correct" cell type in skin (outer root sheath keratinocytes), based on what is known about mouse keratin 6 expression (Heid *et al*, 1986). In our comparative analysis of keratin 6 expression in normal mice *versus* K6-driven ODC expression in transgenic mice, we also observed that expression of both genes begins at the same time after birth (approximately 12 d). Our time course analysis (every 2 d after birth) of both skin histology and expression of K6 and ODC in this transgenic model indicated that the first evidence of follicular cyst development was observed at 12 d after birth, coincident with the onset of both endogenous K6 expression and ODC overexpression. Clearly, ODC overexpression due to activation of the K6-driven transgene drives the development of abnormal skin histology and hair follicle dysfunction in this model.

Important tests of the idea that ODC activity is a regulator of hair follicle function involved the use of a highly specific inhibitor, DFMO, either to prevent the onset of ODC overexpression or to reduce established overexpression to more normal levels. Administration of this drug immediately after birth, before transgene expression was turned on, was very effective in preventing high levels of ODC expression and hair loss. Somewhat surprisingly, administration of the drug after high levels of ODC transgene expression had been established (and hair loss was complete) resulted in reactivation of hair growth, although a completely normal hair coat was not achieved. Reappearance of normal follicles was accompanied by shrinkage and actual disappearance of some of the follicular cysts characteristic of adult transgenic mice. This result suggests that at least some cells in the cysts retain stem cell potential. We conclude from both sets of DFMO experiments that intracellular polyamine levels (especially putrescine) act as a molecular switch regulating the behavior of outer root sheath keratinocytes of the hair follicle: High levels support proliferation and suppress differentiation, whereas low levels are permissive for differentiation but not permissive for proliferation. In normal mice, polyamine levels would fluctuate in response to transient elevations of ODC caused by extracellular factors produced either locally or systemically, whereas the constitutive overexpression of ODC in transgenic mice would eliminate fluctuations in polyamine levels and abrogate exogenous control of follicular keratinocyte growth and differentiation.

Although there are numerous mutations that cause hair loss in mice, the identity of the genes involved is not known in most cases (Sundberg, 1994). Interestingly, mutations in another gene involved in ornithine metabolism, ornithine transcarbamylase, are responsible for the "sparse fur" phenotype (Doolittle *et al*, 1974; DeMars *et al*, 1976). In transgenic mice, overexpression of a sheep wool keratin gene caused cyclic hair loss and regrowth (Powell and Rogers, 1990). In addition, transgenic mice overexpressing the

Table I. DFMO Prevents the Increase in ODC and Polyamine Levels in Transgenic Mice[a]

Treatment	ODC (U/mg Protein)	Polyamine (nmol/mg DNA)		
		Pu	Spd	Sp
DFMO continuous	≤0.01	29.8	844	712
DFMO 3 wk, then off 12 wk	6.63	477	1212	323

[a] The mice depicted in **Fig 5*a*** were euthanized, and extracts of dermis were analyzed for ODC activity and polyamine levels as described in *Materials and Methods*. Pu, putrescine; Spd, spermidine; Sp, spermine.

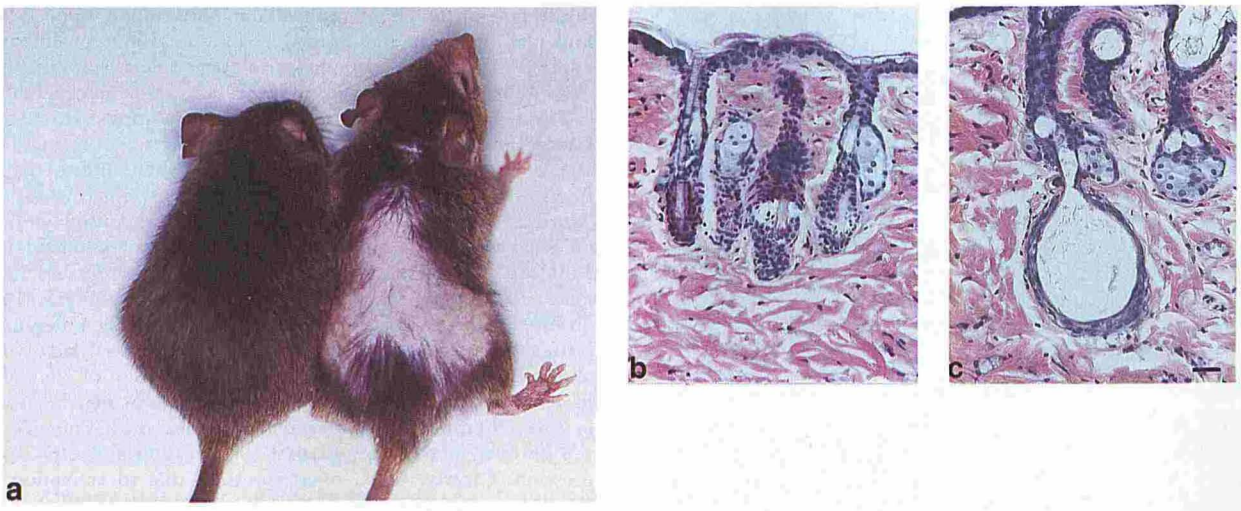

Figure 5. DFMO prevents hair loss. Transgenic mice who were administered DFMO either continuously (*a*; *left*) or only for the first 3 wk of life (*a*; *right*) were euthanized at 15 wk of age, and their skins were examined histologically and for transgene expression. *b*, hematoxylin and eosin–stained section of skin from the mouse administered DFMO continuously; *c*, section from the mouse administered DFMO for 3 wk after birth, then switched to regular drinking water. Note the presence of normal hair-containing follicles in *b* and a developing follicular cyst in *c*. *Scale bar*, 100 μm.

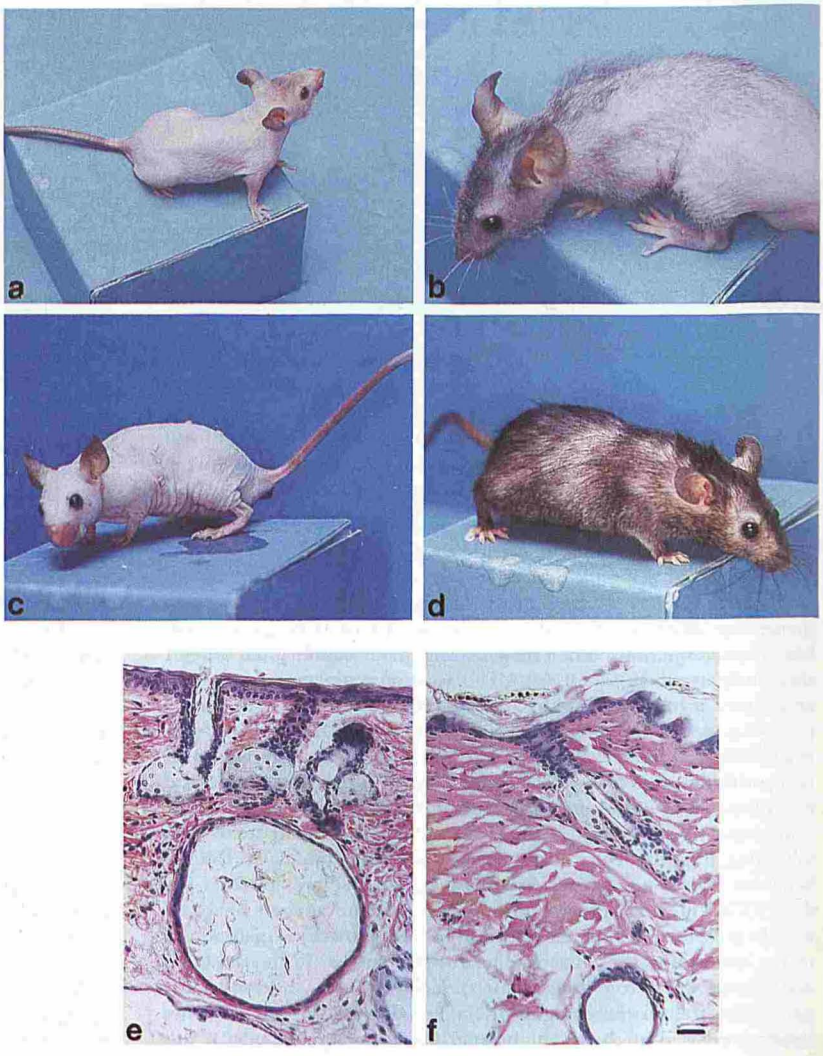

Figure 6. DFMO reactivates hair growth in transgenic mice with complete hair loss. Forty-five-day-old transgenic mice were either maintained on their regular drinking water (*a*,*c*) or switched to drinking water containing 1% DFMO (*b*,*d*). Mice were photographed 17 d (*a*,*b*) or 67 d (*c*,*d*) later. Mice pictured in *c* and *d* were then euthanized, and skins were analyzed histologically (*e*,*f*) and for transgene expression (**Table II**). Note the presence of a normal follicle with hair in the skin from the mouse administered DFMO (*f*). *Scale bar*, 100 μm.

Table II. DFMO Reduces the Elevated ODC and Polyamine Levels in Transgenic Mice[a]

Treatment	ODC (U/mg Protein)	Polyamine (nmol/mg DNA)		
		Pu	Spd	Sp
None	22.5	1020	2134	545
DFMO, 9.5 wk	1.4	49.6	715	795

[a] The mice depicted in **Fig 6*c,d*** were euthanized, and extracts of dermis were analyzed for ODC activity and polyamine levels. Abbreviations as in **Table I.**

transforming growth factor–related polypeptide BMP-4 driven by a K6 promoter exhibit a partial hair-loss phenotype (Blessing *et al*, 1993). The enzymatic activity of ODC in transgenic dermis can be effectively inhibited by a nontoxic (1%) concentration of DFMO in the drinking water. Other approaches such as an inducible transgenic expression system (Furth *et al*, 1994) could also be used to regulate ODC expression levels. Using such models, it should be possible to study the mechanisms involved in the loss of normal hair follicles and the morphogenesis of new follicles, as well as the extrinsic factors that regulate these events.

Finally, does ODC regulate the hair follicle structure and function in other mammals, including humans? Because DFMO is being actively evaluated as a cancer chemopreventive agent, there have been several long-term chronic toxicity studies of DFMO in both animals and humans. Takigawa *et al* (1983) reported that the only side effect of 1% DFMO in the drinking water in mice undergoing long-term anti-carcinogenesis experiments was "severe retardation of hair growth." A 1-year study of daily oral administration of DFMO to dogs and rats reported moderate to severe dermatologic reactions, including alopecia, dermatitis, and conjunctivitis (Crowell *et al*, 1994). In humans, the dose-limiting toxic effect of this compound is ototoxicity (Love *et al*, 1993), which is usually caused by chemically induced damage to the outer hair cells of the inner ear (Harrison, 1988). The finding that hair follicles and inner-ear hair cells are among the most sensitive cell types to ODC-targeted drug treatment suggests that polyamines are critically important molecules in regulating the growth and functioning of these specialized tissues. Defects in polyamine metabolism, therefore, may underlie some abnormalities of skin and hair growth in humans. If so, polyamine-based therapies may have potential for the treatment of such diseases.

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