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Transgenic mouse model of malignant skin melanoma

(skin grafting/wound healing/growth factors/invasion/metastasis)

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ABSTRACT **Tyr-SV40E transgenic mice are specifically susceptible to melanoma due to expression of the oncogene in pigment cells. Mice of the more susceptible lines die young of early-onset eye melanomas, when skin melanomas are still infrequent and benign. To surmount this obstacle, skin from donors of two high-susceptibility lines was grafted to Tyr-SV40E hosts of a low-susceptibility line of the same inbred strain, thereby enabling the skin to outlive the donors and continue to grow in immunocompetent but tolerant hosts. Unexpectedly, donor pigment cells in all the grafts soon selectively proliferated close to areas of greatest wound healing, forming a dense black tracery, especially at the outer rim of the grafts. These lesions slowly grew radially within the grafts, producing irregular greyish patches. Local vertical thickenings then appeared and developed into small melanomas, which soon ulcerated through the epidermis. The tumors rapidly enlarged and became deeply invasive. Discrete black nevi also arose, with many becoming larger and distinctly blue, but those not near areas of pronounced wound healing did not progress to malignancy. In this first series, malignant melanoma resulted in all the grafts from the more susceptible of two donor lines and in some grafts from the other line. Distant metastases occurred in some cases from each line. Most tumors were hypomelanotic and heterogeneous, with lobes or areas differing in melanization. The results strongly suggest that growth factors and cytokines—known to be produced in wound repair—are triggering the growth and malignant conversion of these genetically susceptible melanocytes and that in the graft situation we are merely witnessing a caricature—a usefully exaggerated manifestation of the true events underlying the genesis of melanomas. The striking resemblance to the human malignancy, the genetic uniformity and different susceptibilities of the transgenic lines, and the experimental possibilities in the grafted mice all make them an excellent model of the disease.**

The recent record reduction in the earth's ozone layer (1) blocking the sun's ultraviolet rays may worsen the prediction made in 1987 (2) that 1 in 90 Caucasians in the United States will develop skin melanoma by the turn of the century. In recognition of the already rapidly rising incidence of this disease—second only to lung cancer—a National Institutes of Health Consensus Development Conference on melanoma was convened in 1992 (3). Among its recommendations for future research objectives was "to define the basic biology of melanoma by developing transgenic mouse models" (3). We report here that we have produced a suitable model in which there is a striking resemblance to the human malignancy and in which candidate causal or therapeutic agents can be experimentally tested.

We have described (4) Tyr-SV40E transgenic mice of the standard C57BL/6 strain in which the transgene is comprised of the simian virus 40 (SV40) early region, including the large tumor antigen (T antigen) transforming gene, under the transcriptional control of the tyrosinase promoter expressed in pigment cells. Melanocytes in the hair follicles of the mice are hypomelanotic and the hairs, to which these cells contribute pigment granules, are lighter than normal as a result (5, 6). With increasing age, the skin itself becomes slightly darker due to melanocytic hyperplasia in the dermis. Localized foci appear as small flat black macules, some of which enlarge and thicken to become moles or nevi.

Three classes of neoplasms have occurred: eye melanomas (4, 7), various other nonskin tumors associated with widespread distribution and hyperplasia of neural crest-derived pigment cells (5), and skin melanomas (4). Different inbred lines of the mice, descended from separate founders, all express the T antigen to a degree specific for each line, as seen in the coat color reduction characteristic of each. They differ correspondingly in onset and progression of eye melanomas and were expected to differ in susceptibility to skin melanomas. At the extremes, early and rapidly growing eye tumors arise in mice of lines with lighter grey coats, while eye tumors are late and slow-growing in mice of very dark grey lines. Early eye tumors are fatal at a young age, due to invasion and metastasis; skin melanomas are then still infrequent and usually benign (4). The ultimate capacity of the transgenic skin melanocytes for malignancy was nevertheless demonstrated by establishing the cells in culture soon after birth and obtaining tumors after subcutaneous injection into immunodeficient nude (*nu/nu*) hosts (8, 9). However, cell culture and injection do not reflect the genesis and biology of a melanoma in the skin. Some experimental intervention was therefore needed.

The strategy we have devised is to circumvent the lethality of eye melanomas in the more susceptible transgenics by grafting skin from them to low-susceptibility young adult transgenic hosts. The skin can thus outlive the donor and grow for a long period in a histocompatible C57BL/6 host tolerant of the SV40 T antigen, which is known to be immunogenic (10). Such hosts have the further advantage that their skin (unlike that of nude mice) is structurally similar to the donor skin; moreover, they would be capable (unlike any immunodeficient hosts) of responding to tumor-associated antigens.

While the expectation of obtaining malignant skin melanomas was indeed fulfilled, the graft experiment unexpectedly disclosed many clues suggestive of the biological and molecular mechanisms underlying formation and progression of these tumors.

MATERIALS AND METHODS

A 1-cm disc of full-thickness dorsolateral body skin from a killed donor was scraped free of underlying fat and applied to the trunk of an anesthetized host after removal of a slightly larger piece of host skin. The graft was covered with tulle gras and the trunk was wrapped in moistened plaster-impregnated gauze as described (11). Skin was grafted from female to either sex, or from male to male. Bandages were removed

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Abbreviations: SV40, simian virus 40; T antigen, large tumor antigen.

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after 10 days. Donors were line 8 transgenic hemizygotes (*Tag*/−) (with light grey coats) or line 9 (darker grey) homozygotes (*Tag*/*Tag*); hosts were line 12 *Tag*/− (very dark grey) (Fig. 1). Mice of these lines have only one or two transgene copies in hemizygotes and their differences in transgene expression are attributable to differences in the chromosomal site of integration. Skin was also grafted from transgenics to wild-type C57BL/6 and from wild type to wild type or to transgenic mice of line 12. Donors were 12–22 wk old and hosts were 10–14 wk old.

RESULTS AND DISCUSSION

Melanocyte-Specific Destruction in Transgenic Skin Grafted to Wild-Type Hosts. Melanocytes were selectively slowly destroyed in all nine transgenic grafts to wild-type hosts, while other cells in the donor skin and hair follicles remained intact (Fig. 2). This reflects the presence of some T antigen on the cell surface (12), apart from its chief localization in the nucleus (see figure 2 in ref. 9). The result also demonstrates that the tyrosinase promoter element of the transgene (4) has effectively restricted expression in the skin to pigment cells. The pigment cells remained viable in grafts between wild-type mice, and from wild-type mice to line 12 transgenics, except for infrequent local injury leading rapidly to white patches or isolated white hairs. In contrast, the immunologically mediated loss of the transgenic pigment cells in wild-type hosts began late and required over a year for completion (Fig. 2). The earlier melanocytic destruction in line 8 than in line 9 grafts is consistent with greater transgene expression in line 8 (Fig. 1). Of special interest in grafts from transgenics to wild-type was the more rapid blanching of hairs—probably indicative of greater cytotoxic T-cell activity—at the graft margins than in the interior of the grafts.

Melanoma Induction by Wound Healing in Transgenic Skin Grafted to Transgenic Hosts. Pigment cells persisted, as expected, in grafts from high-susceptibility to low-susceptibility transgenics; hence, donor-type hair color was maintained (Fig. 3). However, a dramatic and unexpected change became evident in the skin within the first few weeks after grafting. At the outermost rim of all the grafts, a blackened edge (Fig. 4*a*, arrowheads) comprised of a dense brushwork of pigment cells arose and became more pronounced with time, especially in skin from line 8. The pigmented rim, just medial to the scar surrounding the graft, was discontinuous macroscopically where the scar was not readily apparent. A dense black tracery also arose in the interior of some of the grafts (Fig. 4*e*, arrowheads).

FIG. 1. Coat color differences in donor and host lines of C57BL/6 mice. Left to right: Tyr-SV40E transgenic line 8 (*Tag*/−), line 9 (*Tag*/*Tag*), and line 12 (*Tag*/−) mice, compared with a wild-type control.

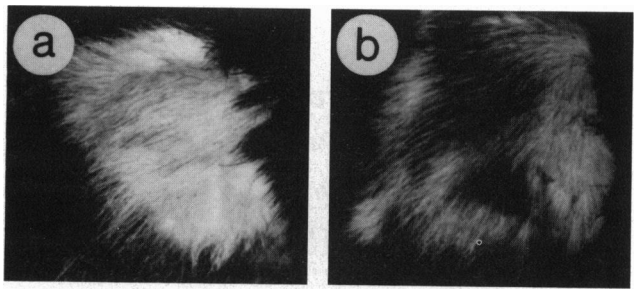

FIG. 2. Selective destruction of pigment cells at different rates in two lines of transgenic (Tyr-SV40E) C57BL/6 skin grafted to wild-type C57BL/6 hosts. (*a*) Line 8 (*Tag*/−) donor skin 53 wk after grafting. (*b*) Line 9 (*Tag*/*Tag*) donor skin 55 wk after grafting.

Although melanocytes are normally quiescent and undergo little mitotic division in the skin of adult mice (13), it was obvious under the dissecting microscope in the present case that the number of these cells had greatly increased in the blackened areas. Wound healing and tissue remodeling are well known to involve an array of growth factors and inflammatory mediators contributed by various cell types in the skin and by cytokines released from immigrant hematopoietic cells (14). It seems likely that the profuse and localized melanocytic proliferation in the grafts is a response to short-range mitogens among the factors associated with wound healing. This specific reaction was not seen macroscopically in wild-type skin grafted to wild-type or to line 12 transgenic hosts. The transgene thus seems to have significantly lowered the threshold for responsiveness to mitogenic factors.

Much later, the hyperpigmentation began to spread in the plane of the skin and became more diffuse, yielding a patch with irregular borders and variable grey coloration. An example at the graft margin (Fig. 4*b*, large arrowhead) is shown expanding medially at 45 wk after grafting. The case with black markings in the interior at 45 wk after grafting (Fig. 4*e*) has by 58 wk (Fig. 4*f*) extended radially across the center of the graft.

Within such radially expanding areas, one or more local thickenings arose, signaling vertical growth. These became early melanomas. Ulceration through the skin surface occurred surprisingly soon thereafter, and growth became more rapid. The disseminating lesion indicated near the graft margin in Fig. 4*b* has formed an ulcerating tumor, shown in Fig. 4*c* (large arrowhead), a mere 2.5 wk later. The spreading patch in Fig. 4*e* has developed three small melanomas within 13 wk, and one (Fig. 4*f*, arrowhead) has started to ulcerate. In still another case (Fig. 4*d*, arrowhead), an ulcerated melanoma has developed within a greyish patch previously derived by horizontal growth from the hyperplastic margin.

FIG. 3. Skin graft from a transgenic donor of line 8 (*Tag*/−) to a transgenic host of line 12 (*Tag*/−), 12 wk after grafting, showing the light grey hair pigmentation characteristic of the donor line.

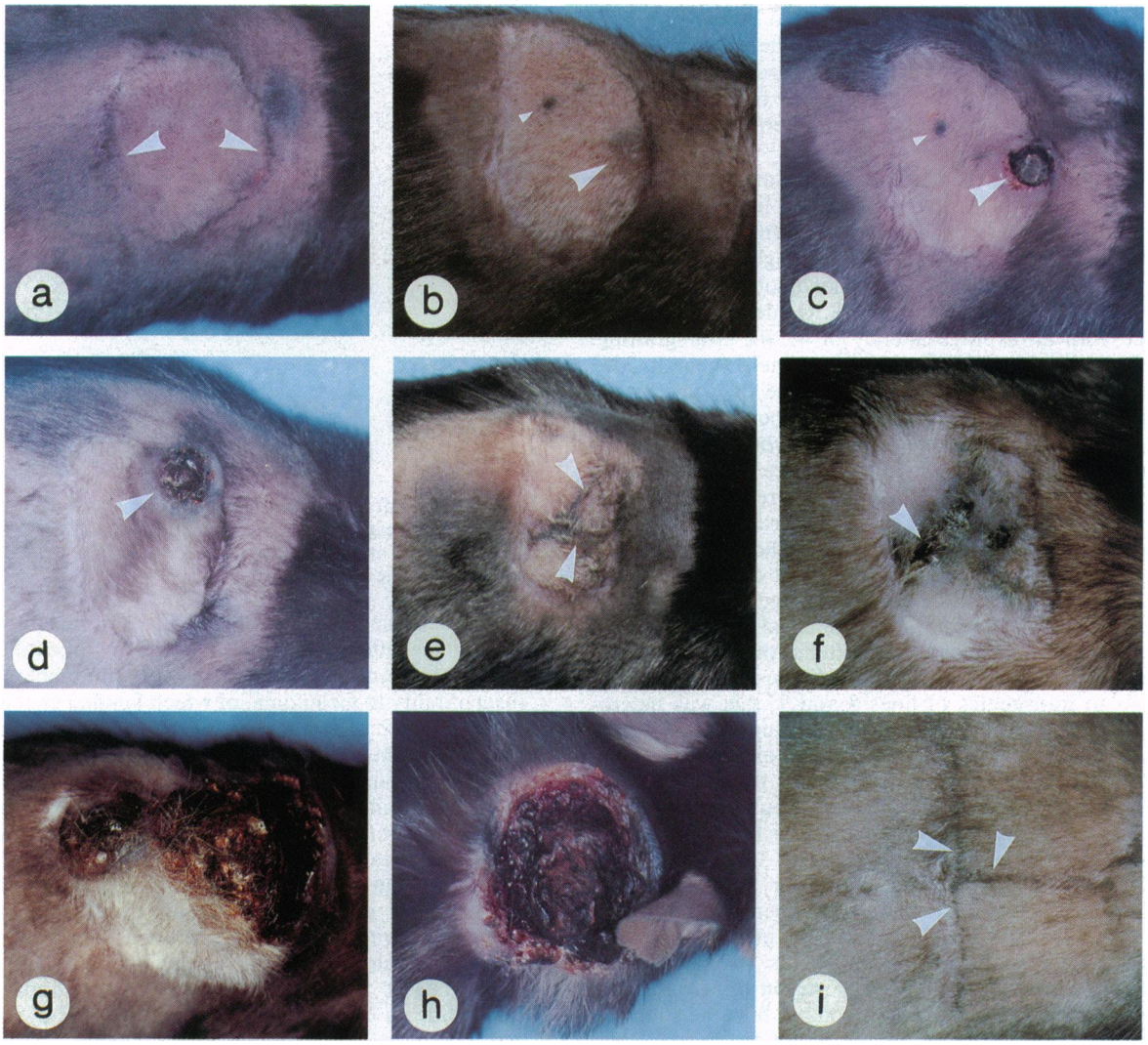

FIG. 4. Effects of grafting (*a–h*) or wounding (*i*) transgenic skin. The experimental area has been shaved. Skin grafts are from homozygous (*Tag/Tag*) donors of line 9 (*a* and *d*) or hemizygous (*Tag/−*) donors of line 8 (*b*, *c*, and *e–h*); all hosts are from line 12 (*Tag/−*). (*a*) Typical early blackening at the graft margin due to a marked increase in pigment cells close to the site of wound healing. (*b* and *c*) Progressive changes in skin of a single graft, culminating in melanoma. (*d*) Another example of similar changes arising near the graft margin and leading to melanoma. (*e* and *f*) Progressive changes arising in the interior of another graft and resulting in three melanomas. (*g* and *h*) Two independent cases of advanced melanomas occupying most of the grafts. (*i*) Skin wounding of a line 9 (*Tag/Tag*) mouse, without grafting, has resulted in melanocytic hyperplasia associated with wound healing. See text for details.

Isolated flat blackish macules also occasionally appeared within the grafts, just as they arise spontaneously in ungrafted skin with increasing age of the transgenics. The black macules often slowly enlarged in diameter and thickness, becoming equivalent to human moles or nevi, with regular borders and uniform coloration. Some nevi became still larger in the course of the experiments and were then deep blue in color, thus resembling human blue nevi. In Fig. 4 *b* and *c*, the indicated blue nevus (small arrowhead) is situated near a small macule that did not increase in size. These mouse blue nevi may sometimes go on to become early melanomas, as observed in ungrafted transgenics (see figure 3 in ref. 4). In the present experiments, none of the solitary nevi distant from areas of dense melanocytic hyperplasia became malignant. However, some that were close to or within such areas seemed capable of progression to malignancy (see ref. 15).

Two examples of more advanced malignant melanomas are shown in Fig. 4*g* (at 53 wk after grafting) and Fig. 4*h* (at 47 wk after grafting). The area of white hairs seen in Fig. 4*g* was present soon after grafting and was probably caused by local destruction of pigment cells during handling and grafting of the skin. Absence of tumor at the graft center in Fig. 4*h* may have resulted from local regression.

It is noteworthy that all melanomas were strictly confined within the grafts. This is surprising because some of the tumors (e.g., in Fig. 4*h*) had metastasized to distant sites and because small numbers of normal melanocytes can move across a wound for short distances in ordinary nontransgenic skin grafts (16). However, the hyperplastic transgenic melanocytes were found to be losing their dendrites (see figure 1*f* in ref. 15) and this may have contributed to a diminished ability to migrate. Radial expansion of the lesions may in fact depend more on increased cell proliferation and invasion of the extracellular environment than on active cell migration.

While the melanomas themselves were all at first heavily pigmented, most later became hypomelanotic or amelanotic, except for one very black tumor (obtained from the case in Fig. 4*f*), which has remained black during serial transplantation into nude as well as into line 12 hosts. Many of the tumors were in fact phenotypically heterogeneous, with lobes or distinct areas of relatively greater or lesser pigmentation. This heterogeneity may reflect either polyclonal origin or cellular diversification and selection.

The tumors were all deeply invasive and three had metastasized to lymph nodes and lungs. When the experiment was terminated, the primary tumors varied greatly in size and progression; therefore, the true frequency of metastasis and the full range of possible target organs remain to be determined.

Our first graft series (Table 1) revealed a marked difference in melanoma inducibility in the two donor lines. Despite the small numbers, the difference is consistent with the more pronounced phenotypic effects of the transgene on coat color and on precocity and severity of eye melanomas in line 8 than in line 9. Group A grafts, from line 8, yielded melanomas in 5/5 mice with an average latency of 46 wk after grafting. Group B grafts, from line 9, developed melanomas in 3/12 mice with an average latency of 51 wk. The 9 negative grafts in group B all exhibited localized melanocytic hyperplasia near the wound but had not yet spread beyond this; at 63 wk after grafting, 3 of the negative mice were killed for histological examination and grafts from the remaining 6 were retransplanted to new line 12 hosts for further observation.

Wound Healing in Transgenic Skin Without Grafting. To learn whether wound healing alone might obviate grafting, two slivers of full-thickness trunk skin were excised from anesthetized transgenic mice (and controls) in the form of a cross so that wounding might be greater at the intersection of the two cuts. Wound repair elicited conspicuous nearby melanosis in transgenics of lines 8 and 9; this was macroscopically visible only where an easily apparent scar was formed (Fig. 4*i*, arrowheads), as in the case of skin grafts (Fig. 4*a*). Wounding alone has thus far failed to result in skin melanomas in high-susceptibility transgenics before they succumb to eye melanomas. The melanosis reaction was not externally apparent after wounding of line 12 transgenics or of C57BL/6 wild-type controls. However, histological examination revealed some melanocytic hyperplasia in line 12 skin near the scar tissue, in excess of the moderate amount that occurs with age in transgenic controls.

Origin of Melanomas from Melanocytes in the Grafts. The origin of the melanomas from graft and not host melanocytes was clearly demonstrated by Southern blot analysis of DNA based on differences between donor and host lines in the chromosomal site of transgene integration (Fig. 5). Thus, after *Eco*RI digestion of DNAs, and electrophoretic separation and transfer of the fragments, DNA hybridization revealed that the skin melanoma in Fig. 4*h* and a lung metastasis of that same tumor (both bracketed in Fig. 5) were from the graft (line 8 in this case) and not the host (line 12); in the last lane, the same is seen to be true of the skin melanoma shown in Fig. 4*c*.

Melanoma Induction in Grafted Transgenic Mouse Skin as a Model of the Human Malignancy. We propose that the strategy of grafting skin from high- to low-susceptibility Tyr-SV40E inbred mice provides an excellent analytical and experimental model of the human disease, for the following reasons: (*i*) The kinds and sequence of externally apparent lesions are remarkably similar to those described in human melanomagenesis (17); histological examination of our mouse material has disclosed further similarities and has reinforced the same conclusion (see ref. 15). (*ii*) Genetic susceptibility plays a strong potentiating role in our animals and in human familial cutaneous melanoma (18, 19), albeit not through SV40 in the latter. The different levels of susceptibility in our transgenic mouse lines may presage differences in human susceptibility to melanoma, of which familial incidence is the extreme. Such differences could be based on genetic modifiers or on heterogeneity of gene structure or function. Sporadic melanoma, whether in mouse or human, may depend on an initiating genetic change in some somatic cells. (*iii*) Skin grafting is not a literal basis for melanoma. Rather it is here a caricature—a useful exaggeration of the true underlying but generally obscure events—enabling those events to be identified. The association of some human melanomas with an earlier severe blistering sunburn may exemplify analogous wound-healing events in initiating melanoma, apart from the role of ultraviolet-induced DNA damage. Data on various growth factors and cytokines and their receptors in human melanomas suggest that the evolution to malignancy may progressively entail increasing responsiveness to paracrine influences and eventual autocrine independence from some of them (see review in ref. 20). (*iv*) Many experimental approaches are feasible only in the grafted transgenic mice. This model allows causality to be distinguished experimentally from mere correlation. For example, a specific gene or agent suspected, on the basis of association, of having a causal role in melanoma could be tested for its ability to increase the low tumor incidence in line 9 grafts (Table 1); or a candidate genetic or other therapeutic agent could be tested for its ability to decrease the high incidence of tumors, or prevent their progression, in line 8 grafts. Moreover, the genetic uniformity within each transgenic line and the uniform strain background allow single variables to be evaluated unambiguously.

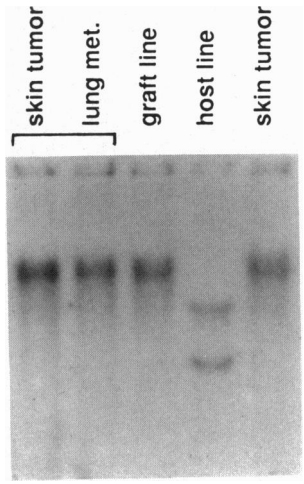

FIG. 5. Graft rather than host origin of melanomas. Southern blot patterns of transgene DNA in a skin melanoma and its lung metastasis (bracketed) and in another skin melanoma are like the graft-line, not the host-line pattern.

The pronounced wound-associated hyperplasia of transgenic but not of normal melanocytes in our mice recalls the early experiments in which repeated abrasion or wounding was first observed to cause epidermal skin tumors in carcin-

Table 1. Malignant melanoma in grafted Tyr-SV40E mouse skin

Exp. group	Transgenic donor line*	Transgenic host line*	No. with melanoma/total (%)	Ave. latency, wk (range)	No. with metastasis
A	8	12	5/5 (100)	46 (42–51)	1
B	9	12	3/12 (25)	51 (44–60)	2

All grafts developed melanocytic hyperplasia in wounded areas. All melanomas caused ulceration and were deeply invasive. Ave., average.

*Line 8 and line 12 mice were hemizygous (*Tag*/−) for the transgene; line 9 mice were homozygous (*Tag*/*Tag*).

ogen-treated mice but not in untreated mice (21–23). Of special interest is the fact that those tumors developed close to the wound edge. More recently, wounding has been found to lead to sarcomas in Rous virus-infected chickens (24) and to skin papillomas in certain transgenic mice (25, 26).

Possibly the introduction of one or more additional transgenes, coding for candidate growth factors experimentally targeted to pigment cells, will result in malignant skin melanomas in our Tyr-SV40E mice without skin grafting. The ability of overexpression of an exogenous growth factor gene to stimulate growth of a target tissue has already been shown in experiments in singly transgenic mice. These have resulted in hyperplasia due to overexpression of transforming growth factor α in the epidermis, liver, mammary gland, or pancreas (25, 27–29). In most cases, the hyperplasia did not lead to malignancy, although coexpression with SV40 T antigen accelerated tumorigenesis in both liver and pancreas (30).

Spontaneous skin melanomas are rare in all laboratory mammals. They occur congenitally, and then regress, in miniature Sinclair swine (31). Repeated long-term exposure of South American opossums to ultraviolet radiation has yielded melanomas, along with other skin tumors (32). Occasional melanomas have arisen after application of carcinogens or of carcinogens and ultraviolet light to the skin of hamsters, guinea pigs, or hairless mice (33–35). The protocol of Berkelhammer and Oxenhandler (36) enabled a higher incidence of skin melanomas to be obtained in C57BL/6 inbred mice by a single topical application of an initiating carcinogen to 4-day-old animals followed twice weekly by a tumor promoter; among the induced skin cancers, 15% were melanomas after a mean latency of 7 mo. Kripke and coworkers (37) succeeded in increasing the frequency of melanomas up to 36% in C3H mice by adding chronic ultraviolet radiation to treatment with an initiator and a promoter; this also shortened the latency to 27 wk. Melanomas were often mixed with other skin tumors such as squamous cell carcinomas, sarcomas, or papillomas.

Apart from the malignant skin melanomas in our grafted Tyr-SV40E mice, no other transgenic mouse models of the human disease have been reported. Transgenic mice of mixed strain background with the *ret* oncogene controlled by metallothionein promoter sequences, which are not specific for pigment cell expression, have developed tumors of various organs but no malignant skin melanomas (38). The melanomas in Tyr-SV40E transgenics, unlike those induced by skin exposure to carcinogens or ultraviolet light, are caused by a single agent expressed only in pigment cells and are not accompanied by any other cutaneous tumors. Combined with the marked resemblance to human cutaneous melanomas and the very high incidence in the more susceptible transgenic skin grafts, this is a promising model to facilitate an understanding of the origin and progression of the human malignancy and to explore a rational basis for therapy.

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