Metastatic Cutaneous Melanoma Promoted by Ultraviolet Radiation in Mice with Transgene-initiated Low Melanoma Susceptibility

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Abstract

An inbred-strain (C57BL/6) transgenic (Tyr-SV40E) mouse model of ultraviolet radiation (UVR)-induced metastatic cutaneous melanoma was produced without the use of chemical carcinogens and without resulting in other skin malignancies. Expression of this transgene occurs specifically in melanocytic-lineage cells. In untreated hemizygous mice of transgenic line 12 there are no skin melanomas, and the oncogenic sequence, which is expressed at a very low level, functions solely as a weak initiating stimulus. UVR [including 65% ultraviolet B (280–320 nm wavelength)] supplied the necessary promoting stimulus leading to melanomas. Of various trial protocols, eight were successful and involved exposure of 112 mice for a limited time on each of 3–10 days starting at 2–3 days of age and totalling 1.1–3.7 J/cm² UVR. Fourteen of these animals developed a total of 15 invasive skin melanomas on the head and body, arising between 37–115 weeks of age and, therefore, often after a relatively long latency. The tumors were melanotic and in five of the mice they yielded macrometastases in regional and distant sites. The single most favorable protocol (1.9 J/cm² total UVR, at 0.38 J/cm²/day for 5 days starting at 3 days of age) led to the highest incidence of melanoma (5 of 19 mice) and one of the lowest mortality rates (2 of 19). No melanomas occurred in UVR-treated nontransgenic C57BL/6 mice. Benign skin keratoacanthomas arose and often regressed in treated transgenic as well as nontransgenic mice. This new transgenic mouse model introduces many novel possibilities for experimental analysis of the melanoma-promoting mechanisms of UVR and also of the ability of specific genetic changes to impede or facilitate the UVR effect.

Introduction

The rising incidence of melanoma in the United States, especially among young people, is thought to be due in part to increased recreational exposure to UVR³ in sunlight (1, 2). Experiments on mice treated with UVR and chemical carcinogens have demonstrated that UVR may lead to melanoma by acting either as an initiating or a promoting stimulus, depending upon the protocol (3). Familial and epidemiological lines of evidence suggest that in human melanoma UVR may more often be acting in a tumor-promoting capacity, with genetic susceptibility factors providing the tumor-initiating potential. Genes influencing DNA repair, the growth and cycling of cells, or skin pigmentation, may be among those with direct or indirect roles in proneness to melanoma (4–6).

We were interested in the possibility of producing a convenient experimental model of UVR-induced malignant melanoma in our Tyr-SV40E transgenic mice, which are specifically melanoma-susceptible (7). Transgene expression in these animals is governed by a transcripitional control region of the murine tissue-specific tyrosinase gene, which therefore activates the oncogenic sequence of the transgene in melanocytic-lineage cells. The mice all have the same standard inbred-strain (C57BL/6) background but they comprise separate inbred lines, each of which is descended from a single fertilized egg injected with transgene DNA. Mice of the different lines have characteristic levels of transgene expression, due chiefly to transgene integration into different chromosomal sites. As a result, they differ in the degree of melanoma susceptibility. Even in the most susceptible lines (e.g., line 8), spontaneous skin melanomas are rare and do not progress beyond an early stage (7). Skin melanomas have never been seen in any of the lines with relatively lower transgene expression, even after skin wounding (8). Spontaneous eye melanomas occur early and are fatal in the high-susceptibility lines but are infrequent and arise very late in mice of the least susceptible line (line 12; Ref. 7). By crossing line 12 transgenic homozygotes with nontransgenic C57BL/6 mice, the marginally susceptible hemizygotes of line 12 were derived for use in the present experiments. Eye melanomas are rarely evident in these hemizygous animals before 2 years of age and progress slowly after they appear.

The ability of UVR to transform Tyr-SV40E skin melanocytes was first shown by irradiation of the cultured pigment cells from a high-susceptibility line (line 9). After s.c. transfer to transgenic hosts, the cells generated melanomas (9), UVR of neonatal mice of that same high-susceptibility line resulted in nevi and early melanomas in the skin (10). As those mice develop fatal eye melanomas, skin containing the lesions was grafted to low-susceptibility line 12 hemizygous hosts, where some of the lesions became malignant melanomas. The objective in the present study was to obtain melanoma progression and metastasis after UVR without skin grafting, therefore without the added melanomagenic stimulus of factors involved in wound-healing of grafts (8), and also without applying chemical carcinogens. We have, therefore, undertaken UVR treatment of low-susceptibility transgenic neonates that are line 12 hemizygotes. As predicted (10), appropriate choices of the transgenic line and other variables have now yielded a favorable incidence of melanoma, with metastasis, in relatively long-lived mice of minimal melanoma susceptibility.

Materials and Methods

Mice. Experimental mice were all inbred Tyr-SV40E (C57BL/6 strain) transgenic hemizygotes of line 12 (7). Controls were nontransgenic C57BL/6 mice. All of the animals were housed in clear plastic cages with opaque filter tops under standard conditions of temperature, humidity, and air flow. The room was illuminated for 12 h/day by fluorescent tubes from which no UV component was detectable at the distance to the animals. UVR. Experimental and control mice were exposed to UV light in an enclosed wooden chamber 60 cm wide, 126 cm long, and 83 cm high, fitted at ceiling level with a bank of four F40 Philips UVB sunlamps. This lamp replaces the Westinghouse FS40 lamp and has a similar spectral emission curve and a continuous spectrum in which 65% of the output is in the UVB range (280–320 nm wavelength) with a peak emission of 313 nm (11). Mice were placed 20 cm from the sunlamps in a molded opaque plastic tray with 10 parallel slots 1.5 cm wide and 1.1 cm deep. This provided adequate restraint
while usually shielding only the lower legs and entire ventrum. UVB irradiance was measured immediately before each irradiation by means of an SED240/UVB-1/W detector connected to an IL1700 radiometer (International Light, Newburyport, MA). Dose rates (irradiance) varied between 0.36 and 0.46 mW/cm²; exposure times were chosen to deliver the desired dose. Irradiated mice were examined biweekly for presence of skin lesions and tumors.

**Results**

**Incidence of UVR-induced Cutaneous Melanomas.** UVR protocols differed with respect to age of the mice at first treatment (2 or 3 days of age, counting the day of birth as day 0), number of days of treatment (1–10 days), and dose per day (0.14–0.59 J/cm²). The gradual growth of the pelage during the ages that were spanned by treatments means that an indeterminate amount of the administered UVR did not actually reach the skin, especially in protocols extending as long as 10 days. Thus, the total dose of UVR to the skin itself is likely to have been less than indicated in Table 1 in all of the cases. A number of treatment regimens had to be explored to learn which eventually yielded the highest viability and melanoma incidence. In general, treatments starting on day 3 resulted in better survival than those starting on day 2 (data not shown). Of a total of 232 treated transgenic mice (109 females, 123 males) in 34 litters, 158 mice (72 females, 86 males), or 68%, survived to weaning age at 4 weeks. Of these survivors, melanomas arose only among mice in the eight protocols listed in Table 1, which involved a total of 112 mice (54 females, 58 males). In comparison with these melanoma-positive protocols, most of the other (melanoma-negative) ones were of shorter duration (1–2 days), and/or they supplied a lower UV dose per day. In a few of the unsuccessful protocols, higher UV doses were used but were soon fatal to most of the treated animals. Treatment with the eight melanoma-positive protocols yielded 83 mice (74%) surviving to weaning age at 4 weeks. (Among untreated mice, ~95% generally survive to weaning.) In the treated group, 80 of the 83 weanlings remained alive to at least 37 weeks of age—the time when the first melanoma appeared. After the mouse in which the last melanoma was detected (at 115 weeks), 28 mice in protocols 1–8 were still alive. They were monitored until 130 weeks of age. The 14 mice with melanoma (Table 2) constitute 18% of the 80 individuals surviving for 37 weeks or longer. The incidence of mice with melanoma may actually have exceeded this level because some of the animals found dead of unknown causes between 37 and 115 weeks were too auto- matically to identify any early melanomas. Two of the 14 mice with melanoma were in protocol 3, 5 were in protocol 6, and 2 were in protocol 8; the other 5 cases occurred singly in protocols 1, 2, 4, 5, and 7.

From the above results, protocol 6 was identifiable as the protocol resulting in one of the lowest mortality rates [2 (11%) of 19 in this trial group] and in the highest incidence of melanoma [5 (26%) of 19; Tables 1 and 2]. In contrast, no melanomas arose in UVR-treated nontransgenic C57BL/6 controls. One group of 21 controls (6 females and 15 males, in 3 litters) received protocol 6; 16 mice were alive at 37 weeks of age and 9 mice were still alive at 115 weeks. Fifty additional controls received total doses of 2.0–3.7 J/cm² UVR. All of them were monitored for over 2 years.

**Locations of Primary Melanomas.** Exposure to UVR sometimes clearly resulted in darkening of the coat in the exposed areas, chiefly on the head and the dorsal and dorsolateral body. This was first noted ~3 weeks after UVR in 5–10% of transgenic as well as nontransgenic animals and persisted for variable periods ranging from a few weeks to ~1 year. UVB has been found to up-regulate tyrosinase enzyme activity and melanin synthesis as well as acting as a mitogen for melanocytes (12). It has been suggested that DNA damage and/or repair of the damage plays a role in UV-induced melanogenesis (13). All of the transgenic melanomas eventually developed within the area in which hypermelanization had been noted (Table 2). Some flat pigmented macules and one or more nevi (including some blue nevi) were observed in 13% of the transgenic mice surviving to at least 37 weeks of age. These were found in various sites on the body but were rare on the tail. Although most of the nevi did not give rise to melanomas, some melanomas were clearly preceded by a blue nevus. No nevi were seen in irradiated nontransgenic controls.

One cutaneous melanoma appeared in each of 13 affected animals; two melanomas occurred in the remaining mouse (Table 2). Examples of the tumors are shown in Fig. 1. Cases 2F-2 and 1W-1 were located on the anterior trunk (Fig. 1, a and d); case 2G-2 projected from the pinna deep inside the ear (Fig. 1e). Five of the 15 tumors were in fact found on or near an ear. These areas have little or no hair to afford protection against incident UVR, and they may also have a relatively large population of extrafollicular melanocytes (14). Three melanomas arose on other parts of the head or face, one occurred on the neck, five were on the body, and one was on the tail at the base. The primary melanomas were chiefly of the nodular type. Fourteen of them were thick and large; 1 of the 2 tumors of case 1U-2 was relatively small when that animal was killed. The tumors were all ulcerated on the surface, either to a slight extent (Fig. 1e) or markedly (Fig. 1, a and d). A striking feature of all the primary melanomas was their darkly melanotic appearance with minor hypomelanotic parts present in some (Fig. 1b).

**Melanoma Metastases.** Animals with a skin melanoma were killed at various times that were chosen to avoid imposing an excessive tumor burden or prolonging apparent ill health. They were examined for macrometastases at necropsy under a magnification of X7; the brain was usually not examined. No samples of lymph nodes

<table>
<thead>
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<th>Table 2 UVR-induced cutaneous melanomas</th>
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<tr>
<td>Mouse code no.</td>
</tr>
<tr>
<td>---------------</td>
</tr>
<tr>
<td>2F-2 M*</td>
</tr>
<tr>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>2A-5 M</td>
</tr>
<tr>
<td>1T-2 M*</td>
</tr>
<tr>
<td>2G-2 F*</td>
</tr>
<tr>
<td>1W-1 F*</td>
</tr>
<tr>
<td>1N-3 M*</td>
</tr>
<tr>
<td>2H-5 M*</td>
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<tr>
<td>1D-4 M</td>
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</table>

*a* Of 80 UVR-treated mice alive at 37 weeks, 28 mice were still alive at 115 weeks.

*b* Melanomas were all located dorsally or dorsolaterally.

*"The primary melanoma had metastasized in each of these cases.

*d* Lympoma was also present.

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Table 1 UVR protocols that resulted in cutaneous melanoma

<table>
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<tr>
<th>UVR protocol no.</th>
<th>Age at start/ no. days (UV dose per day)</th>
<th>Total dose (J/cm²)</th>
<th>No. mice (no. litters)</th>
<th>No. mice alive at 4 wk</th>
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<tr>
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<td>1.8</td>
<td>15 (2)</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>2/5/0.27–0.28</td>
<td>1.1</td>
<td>21 (3)</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>2/5/0.22–0.23</td>
<td>1.1</td>
<td>20 (3)</td>
<td>18</td>
</tr>
<tr>
<td>4</td>
<td>2/4/0.27</td>
<td>1.1</td>
<td>7 (1)</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>3/100/0.37</td>
<td>3.7</td>
<td>16 (2)</td>
<td>16</td>
</tr>
<tr>
<td>6</td>
<td>3/35/0.38–0.39</td>
<td>1.9</td>
<td>19 (4)</td>
<td>17</td>
</tr>
<tr>
<td>7</td>
<td>3/40/0.42</td>
<td>1.7</td>
<td>6 (1)</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>3/30/0.37</td>
<td>8 (1)</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

*a* Age in days, with the day of birth as day 0.

*b* Dose in J/cm², given in one exposure of 10–17.5 min/day.

*"Total 54 females: 58 males.

*"Total 83 alive: 112 UVR-treated = 74% alive at 4 weeks. Of these, 80 mice remained alive until at least 37 weeks after treatment.

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METASTATIC MELANOMA IN UVR-TREATED TRANSGENIC MICE

Fig. 1. UVR-induced melanomas in three transgenic mice. In a, case 2F-2, an ulcerated skin melanoma is located above the left shoulder. In b, the skin melanoma was surgically removed from case 2F-2 and is shown from the ventral aspect. It is densely melanotic except for limited hypomelanotic areas. In c, melanotic macrometastases from the same animal were found in the large left axillary areas. In d, case 1W-1, a melanotic skin melanoma was surgically removed from case 2F-2 and is shown from the ventral aspect. It is densely melanotic except for limited hypomelanotic areas. In e, case 2G-2, a melanotic skin melanoma projects from the pinna in the right ear.

or other organs were collected for histological evaluation if metastases were not clearly seen at this magnification; thus, micrometastases would be undetected. From the fourteen advanced tumors in Table 2 (excluding the small ear tumor in case IU-2), macrometastases were found in a total of five cases (36%).

In two of the 14 mice, the primary tumor was removed surgically, under Nembutal anesthesia, together with a wide margin of surrounding skin. The necropsy was conducted 10 weeks later in one case (2F-2) and 13 weeks later in the other (1T-2). (The surgery was carried out as an adjunct to a larger study in which the effect of excision of a primary melanoma on the growth of its metastases was being investigated in Tyr-SV40E mice of other transgenic lines whose skin melanomas were not induced by UVR.4 At autopsy, operated mouse 2F-2 clearly had metastases in a greatly enlarged left axillary lymph node, in a smaller left brachial lymph node, and in many small foci in both lungs; all of the metastases were largely melanotic and may be compared, in Fig. 1c, with the skin tumor of origin in Fig. 1b. (Histological sections of the primary tumor and a lung metastasis are shown in Fig. 2, a and b.) The other operated mouse, case 1T-2, had small metastases in the left kidney (Fig. 2d) which, like the primary tumor (Fig. 1d), were melanotic. The remaining three (unoperated) mice with macrometastases all had lymph node involvement. Case 2B-2 had a large affected left inguinal lymph node (Fig. 2e) and a small affected left axillary node (Fig. 2f). Case 2D-3 had multiple small metastatic foci in the right axillary lymph node. Case 2H-1 had metastasis in a node near the trachea. All of the macrometastases were melanotic. It is of interest that the affected lymph nodes in 2F-2, 2B-2, and 2D-3 were regional nodes in proximity to the primary tumor located on the same side of the respective individuals. Thus, they may function as draining nodes in which early tumor dissemination can be monitored (15).

Histopathology of Primary Melanomas and Metastases. All primary melanomas were classifiable as malignant on the basis of their penetration into the deep dermis and invasion of s.c. tissues to various extents. As stated above, five of the primary tumors had yielded readily visible macrometastases. Ulceration of the skin surface (Fig. 1, a and d) was common and involved degrees of destruction of the basement membrane and overlying epidermis. Histologically, the changes resembled those in transgenic tumors promoted only by wound healing in skin grafts (16). Melanoma cells in the primary and disseminated tumors were either epithelioid, with round nuclei (Fig. 2e), or spindle-shaped (Fig. 2c), especially in advanced stages of malignancy. The skin tumors as well as their metastases appeared densely black, or largely black with minor hypomelanotic areas (Fig. 1, b and c). They were more often melanotic than the tumors previously obtained in skin grafts from another line (line 8) characterized by a higher level of transgene expression and more rapid malignant progression (17–20). Histologically, the hypomelanotic cells tended to be slightly smaller than the melanotic ones. Many tiny dust-like melanized granules were distinguishable in the cytoplasm of melanotic cells. Conspicuous large black cells (often referred to by others as melanophages) were filled with packed pigment granules and were more numerous in the most melanotic areas (Fig. 2, a, c, e, and f).

Pockets of enlarged spaces in metastasis-bearing lungs are indicative of emphysema (Fig. 2b). In the kidney metastasis shown in Fig. 2d, the tumor has invaded some of the kidney tubules. The enlarged lymph node in Fig. 2e has had all of its lymphoid tissue replaced by proliferating melanoma cells. The smaller lymph node from the same animal (Fig. 2f) still has some lymphoid tissue (seen at the left edge) but the remainder consists largely of melanoma cells.

Nonmelanoma Tumors. Nonmelanoma skin tumors were attributable to the UVR rather than to the transgene. Benign keratoacanthomas were the most frequent type and were found in UVR-treated transgenic as well as nontransgenic animals. Among the 80 transgenic survivors referred to in Table 1, 40% of the mice had from one to four keratoacanthomas on the tail. Most arose 6–8 months after UVR; one-fifth of the lesions regressed spontaneously in 8–20 months after detection. Keratoacanthomas also arose on the tails of 4 of the group of 16 surviving C57BL/6 nontransgenic controls treated with UVR. Only one other skin tumor—a small and questionably dysplastic papilloma—was present in a UVR-treated transgenic mouse.

Four cases of lymphoma were found among the transgenic mice with a skin melanoma (Table 2); none occurred in irradiated controls. Based on histological examination, they represented four decreasingly differentiated (and increasingly aggressive) examples: an early localized lymphoma (in case 1N-3), a lymphocytic lymphoma (in 2H-5), systemic lymphoblastic lymphoma (in 1W-1), and a relatively undifferentiated large-cell lymphoma (in 2G-2).
Discussion

The likelihood that environmental UVR is a contributing cause of human melanoma has stimulated efforts to devise experimental animal models of UVR-induced melanoma. Model animals have included fish, goats, opossums, and mice. The effectiveness of specific wavelengths of UVR has been most extensively analyzed in platyfishswordtail hybrid fish (21). Among mammalian models, the Angora goat has been studied because it develops melanocytic lesions in the most sun-exposed areas of the body that are not covered by dense hair; the lesions become malignant in approximately 2% of the animals. Repeated shearing was attempted but resulted only in benign lesions after a 9-month period (22). South American adult opossums have been experimentally exposed to UVR three times/week for 70 weeks, after the animals were shaved; approximately 6% of those treated developed melanoma. Metastasis was limited to lymph nodes (23). In this species, UVR apparently acts as a complete carcinogen. More recently, newborn opossums, which are developmentally immature and at first hairless, were exposed near the mother’s pouch to a low UVR dose three times/week for 3 weeks. This increased the incidence of melanoma in comparison with treatment of adults. To obtain metastasis in sites other than lymph nodes, melanoma cells from an affected node were cultured and then allografted s.c. to immunologically tolerant neonatal recipients (24).

The availability of inbred strains of mice allows UVR effects to be analyzed in these more experimentally accessible laboratory mammals whose genetic background is uniform. Treatments of mice have heretofore involved various combinations and sequences of chemical carcinogens and UVR. Some treatments have been started a few days after birth (25). It is of interest to note the highest melanoma incidence obtained in any of these protocols. A carcinogen (7,12-dimethylbenz[a]anthracene) was topically applied and used as the initiator, starting at three weeks of age, and was followed by promotion with UVB. When the study was terminated, the animals were 65 weeks old and 6% of all of those treated had developed melanoma (3). A similar overall incidence was obtained in our study, in which 6 (5%) of 112 treated animals (the first six cases in Table 2) had a melanoma by 65 weeks of age. However, in our most successful trial protocol (protocol 6), involving 19 mice treated with UVR, 5 (26%) of 19 developed melanoma. Another of the protocols used by Romerdahl et al. (3) resulted in skin melanomas in 38% of the mice when croton oil was added to the treatment and was applied together with UVB as a copromoter. When croton oil was used as the sole promoter, 25% of animals still developed melanomas; thus, a high percentage of tumors obtained after UVB apparently required croton oil as an accompanying copromoter. Metastasis to regional lymph nodes was observed.

In other experiments, starting at 6–8 weeks of age, an inbred strain of genetically hairless mice was used (26), as in an earlier experiment with noninbred hairless mice (27), to circumvent repeated shaving or chemical depilation of the skin. One initiating topical application of the carcinogen, followed by promotion with UVR three times/week for 30 weeks, resulted in melanomas in 25–33% of the animals (26). No metastasis was reported.

Other skin tumors—particularly papillomas, sarcomas, and squamous cell carcinomas—were frequent in experiments based on the use of chemical carcinogens alone or in combination with UVR (3, 25, 26). These nonmelanoma skin tumors may be attributable to effects of the carcinogens; they are absent in our experimental mice, except for one early papilloma.
In contrast to the lack of such tumors, lymphomas have arisen in four of our transgenic mice that were exposed neonatally to UVR and developed a melanoma (Table 2). Some lymphomas have also been found in mice treated with a chemical carcinogen plus UVR or with a carcinogen alone (26), but the lymphomas often arose in animals without melanomas and may have been due to an effect of the carcinogen. It is noteworthy that lymphomas have been reported as the most frequent additional malignant neoplasm in a large group of melanoma patients in whom the lymphoma incidence was more than 16-fold higher than in the general population after adjustment for age, race, and sex (28). The data were interpreted as indicating that immunosuppression is not an underlying cause (28). However, this may be regarded as an open question in the present experiments with UVR in view of the evidence for an immunological suppressive effect of UVR in mice (29).

The melanomas in our UVR-treated transgenic mice are clearly similar to human cutaneous melanomas in their development, progression, and metastasis. One-half of the mouse melanomas arose more than 1.5 years after treatment (Table 2). This long latency relative to the murine life span is reminiscent of the midlife occurrence of many human melanomas and suggests that UVR may be acting as a weak promoter in both species. The mice provide a standardized and convenient inbred model in which to investigate the promotional effect of UVR in melanoma as well as the efficacy of different UV wavelengths and the role of UVR-induced DNA damage and repair (30), in the absence of other skin malignancies. The induction of only one melanoma in most of the affected mice has the advantage that metastasis is attributable to a specific primary tumor and also that the consequences of its surgical removal on the growth of existing metastases can be determined. Many single-gene mutations of interest are available on the same genetic background (C57BL/6) and may be introduced into Tyr-SV40E mice simply by breeding. Among the questions experimentally tractable in vivo through this approach is the presumed photoprotective role of melanin in the skin. This can now be evaluated through genes known to alter the amount, type, or cellular distribution of melanin.

References
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