Isolation of a Unique Melanogenic Inhibitor from Human Skin Xenografts: Initial *In Vitro* and *In Vivo* Characterization

Jamal Z. Farooqui, Edward Robb,* Steven T. Boyce,* Glenn D. Warden,* and James J. Nordlund
Department of Dermatology, University of Cincinnati College of Medicine, and *Shriner's Burns Institute, Cincinnati, Ohio, U.S.A.

Previously, split-thickness human skin grafted onto athymic mice has been shown to become markedly hyperpigmented, but the factor(s) responsible for this hyperpigmentation had not been isolated. The present study describes the isolation and characterization of a potent melanogenic inhibitor from grafted human skin. Extracts from grafted skin inhibited, in a concentration-dependent manner, tyrosinase activity of normal human melanocytes and of Cloudman S91 murine melanoma in culture. Sodium dodecylsulfate-polyacrylamide gel electrophoresis analysis of extracts from pre- and post-grafted skin demonstrated the presence of a protein doublet of approximately 14 kD exclusively in the post-grafted skin. This protein inhibited both tyrosinase activity and cellular proliferation in a concentration-dependent manner. The inhibition of tyrosinase activity in normal human melanocytes was 53% at 0.5 μg/ml concentration, whereas this inhibition was almost complete in murine melanoma cultures at 1.0 μg/ml. The protein did not inhibit either cellular proliferation or protein synthesis in normal human fibroblast cultures, and therefore may act specifically on melanocytes. Injections of the inhibitor corresponded with a delay and reduction in the quantity of pigment in human skin 2 weeks after grafting. Multiple injections of the inhibitor into the hyperpigmented xenografts (20 weeks after grafting) reversed the hyperpigmentation with no observable inflammatory or toxic responses. The results indicate that hyperpigmented human skin xenografts contain a potent inhibitor of melanogenesis and melanocyte proliferation. *Key words: athymic mice. J Invest Dermatol 104:739–743, 1995*

Previous studies from other institutions [1,2] and from this laboratory [3] have indicated that human skin xenografts on athymic mice undergo marked hyperpigmentation. The pigmentation seems to be greater in meshes or split-thickness grafts than in full-thickness grafts [3]. At present, the exact molecular mechanism of this hyperpigmentation is not known. It was reported recently that the density and size of dihydroxyphenylalanine (DOPA)-positive melanocytes were significantly increased after grafting [3]. Concomitant with this increase, elevated levels of known melanogenic enzymes and melanogenic peptides were also observed [4]. However, the mechanisms by which cellular proliferation and melanogenic activation are stimulated are not known.

To define the molecular mechanisms for this hyperpigmentation, we investigated the molecule(s) responsible for this phenomenon. First, the presence of a potent melanogenic inhibitor present exclusively in the grafted human skin was identified. This inhibitor profoundly blocks both melanogenesis and cellular proliferation in culture. The present study describes the isolation, biochemical characterization, and biologic activity of this melanogenic inhibitor *in vivo* and *in vitro*.

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Reprint requests to: Department of Dermatology, University of Cincinnati, P.O. Box 670592, Cincinnati, OH 45267-0592.

MATERIALS AND METHODS

Skin Grafting Procedures for grafting human skin onto athymic mice (nu/nu, albino Balb/C) have been described previously [3] and were approved by the University of Cincinnati Institutional Animal Care and Use Committee. Briefly, murine skin was excised, and split-thickness skin graft was sutured to wound punctures. At selected times after grafting (usually 12–15 weeks), the grafted mice were sacrificed using an overdose of Nembutol and the grafts were removed.

Extraction of Proteins from Skin Both pre- and post-grafted skin was cut into small pieces after removal of subcutaneous fat. Twenty percent (wt/vol) skin homogenates were prepared in phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride using a motorized homogenizer (Polytron; Brinkman, Westbury, NY). After centrifugation at 2000 $\times g$ for 20 min, the supernatant was collected and centrifuged again at 100,000 $\times g$ for 1 h to obtain clear soluble fraction. Both crude extract and supernatant were filter sterilized, and protein content was determined by the Bradford method [5].

Treatment of Cell Cultures with Skin Extract and Measurement of Tyrosinase Activity The culture conditions for normal human melanocytes [6] from neonatal foreskin and for Cloudman S91 melanoma cells [7] were similar to those described previously.

In a typical cell culture experiment, cells were inoculated at a density of 1.5×10^5 cells into six-well plates containing the respective media for human melanocytes or Cloudman S91 cells. Forty-eight hours later, media in all wells were replaced with fresh media containing increasing concentrations of pre- or post-grafted skin supernatant and/or electroeluted proteins (mean = three per condition). Between 48 and 72 h of the experiment, fresh media containing [^{3}H]tyrosine (1 μCi/ml) plus supernatants or isolated proteins were added to the cultures. The conditioned medium from each well was used to determine the secreted tyrosine

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hydroxylase activity of tyrosinase, according to a modification of the Pomerantz charcoal absorption method as described previously [8–10]. Cell counts per well were determined by Coulter counter. Triplicate wells were used for each experimental condition (mean = three per condition). Responses of cell proliferation by human melanocytes or Cloudman S91 cells were studied by measuring the rates of [^{3}H]thymidine incorporation according to a previous study [7].

Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Electroelution of Proteins from Gel Skin extracts (50 μg protein) were subjected to SDS-PAGE using the discontinuous buffer system described by Laemmli [11] in 15% slab gel. After separation, the proteins were stained with a 0.05% wt/vol solution of Coomassie brilliant blue R-250. In some cases in which the separated proteins had to be electroeluted from the gels, only a part of the gel along with molecular-weight standards was cut out and stained. After destaining, both stained and unstained gels were aligned side by side, and the protein band of interest was excised from the unstained gel for electroelution. Proteins from the excised portions of the gel were eluted in buffer containing Tris (20 mM), glycine (150 mM), and SDS (0.05%). Electroelution was performed in an electrophoretic chamber (Schleicher & Schuell) at 150 V overnight. The fractions were collected, concentrated, and dialyzed by Centricon-3 (Amicon Corporation). The concentrated fractions were then filter sterilized, and protein contents were determined.

***In Vivo* Characterization of Electroeluted Proteins** Proteins electroeluted from SDS gels were analyzed for their effects on hyperpigmented athymic mice (nu/nu) (20 weeks and 2 weeks after grafting). The animals were divided into two groups: the experimental group (A), with five mice, and the control group (B), including two mice. In group A, 50 μl containing approximately 2 μg of protein was injected subcutaneously into a circled area of the graft at weekly intervals. Five such injections were given on the left side of the grafted mice. As an internal control, 50 μl of normal saline was injected on the right side of the same graft. In group B, no injection was given, and this condition provided baseline values for melanocyte counts. Biopsies were done in both groups at time 0 and 1 week after the final injection; specimens were processed for counting DOPA-positive melanocytes and for routine light microscopy to visualize any morphologic changes after the injections. Photographs were taken every week to assess any change of color in the skin graft.

RESULTS

Inhibition of Tyrosinase Activity in Normal Human Melanocytes Treated with Grafted Skin Extract Treatment of normal human melanocyte cultures with increasing concentrations of post-grafted skin extract (ranging from 25 to 100 μg/ml) resulted in an early increase in tyrosinase activity at lower concentrations of skin protein (25 μg/ml), followed by a concentration-dependent inhibition at higher concentrations. Compared with control, approximately 50% of tyrosinase activity was inhibited at 100 μg/ml. Cells treated with pre-grafted skin extract showed no significant inhibition of tyrosinase activity **(Fig 1)**. A similar pattern of inhibition was observed with murine melanoma S91 cells.

Presence of 14-kD Protein in Post-Grafted Human Skin Extracts and Supernatants To identify specific stimulatory or inhibitory proteins associated with pre- and post-grafted skin, we performed SDS-PAGE analyses of crude extracts. As shown in **Fig 2**, there was no major difference between pre-grafted crude extract and supernatant (*lane 1 versus lane 2*) and between post-grafted skin extract and supernatant (*lane 3 versus lane 4*). However, comparisons of pre- and post-grafted skin showed differences in protein patterns. The protein in pre-grafted skin of approximately 50 kD and 66 kD probably represents albumin. Another significant difference was the presence of a doublet of approximately 14 kD present exclusively in the post-grafted skin (*lanes 3, 4*). A fraction extracted with Triton X-100 did not contain this protein, an indication that it is a soluble protein (*lane 5*).

14-kD Protein from Post-Grafted Skin Inhibits Tyrosinase Activity in Normal Human Melanocytes Because the major difference between pre- and post-grafted skin was the presence of a 14-kD band, we studied its effect on tyrosinase activity in normal human melanocytes. This protein was electroeluted from multiple gels and added to the cell cultures at concentrations of 0.25 μg/ml and 0.50 μg/ml. The supernatant from pre-grafted skin was run under identical conditions, and the gel corresponding to the position of 14-kD molecular-weight marker or 14-kD band was cut out and the fraction was electroeluted to ensure that the observed effect was only due to the 14-kD band and was not from the polyacrylamide gel buffer. As shown in **Table I**, the addition of various concentrations of 14-kD protein from post-grafted skin resulted in a concentration-dependent inhibition of tyrosinase activity. This inhibition was 53% at 0.5 μg/ml concentration. No significant inhibition was observed when electroeluted fraction from pre-grafted skin was added to the cultures.

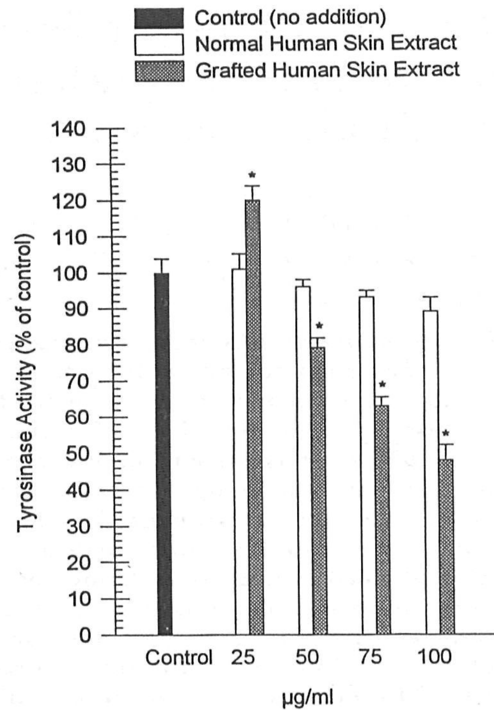

Figure 1. Inhibition of tyrosinase activity in normal human melanocytes in the presence of extracts from post-grafted human skin. Cells were initially inoculated at 1.5 × 10^5 cells/well (in six-well dishes) for 48 h. Thereafter, the medium was removed and fresh medium with various increasing concentrations of pre- and post-grafted skin extracts (ranging from 25 to 100 μg/ml) was added. Two such treatments were given every 48 h. During the last treatment, the medium was supplemented with 1, 3[^{3}H]tyrosine (1 μCi/ml). After 24 h, the medium was collected for tyrosinase assay and the cells were counted by Coulter Counter. The enzyme activity was calculated in units of cpm/10^6 cells and expressed as a percent of the control value. Values are the mean of three determinations ± SD. $^*p < 0.05$ (Student t test).

14-kD Protein Inhibits Tyrosinase Activity and Cell Proliferation in Murine Melanoma Cells The effect of 14-kD protein isolated from post-grafted skin on melanogenesis in murine melanoma cells was also determined. The cell cultures were treated with different concentrations of 14-kD protein ranging from 0.25 to 1.0 μg/ml. As shown in **Fig 3**, a concentration-dependent inhibition of tyrosinase activity was observed, and the inhibition was almost complete (90%) at 1.0 μg/ml concentration. A minimal inhibition of tyrosinase activity was seen in the presence of the fraction electroeluted from the pre-grafted skin. Cell proliferation ([^{3}H]thymidine incorporation) was also reduced significantly in the presence of 14-kD protein **(Fig 4)**.

To examine whether the 14-kD protein isolated from post-grafted skin was specific for melanogenic enzyme and was not a general metabolic inhibitor, we studied its effect on cultured normal human fibroblasts. Incubation of normal human fibroblasts with up to 10-fold higher concentration of the protein (0.25 to 2.5 μg/ml)

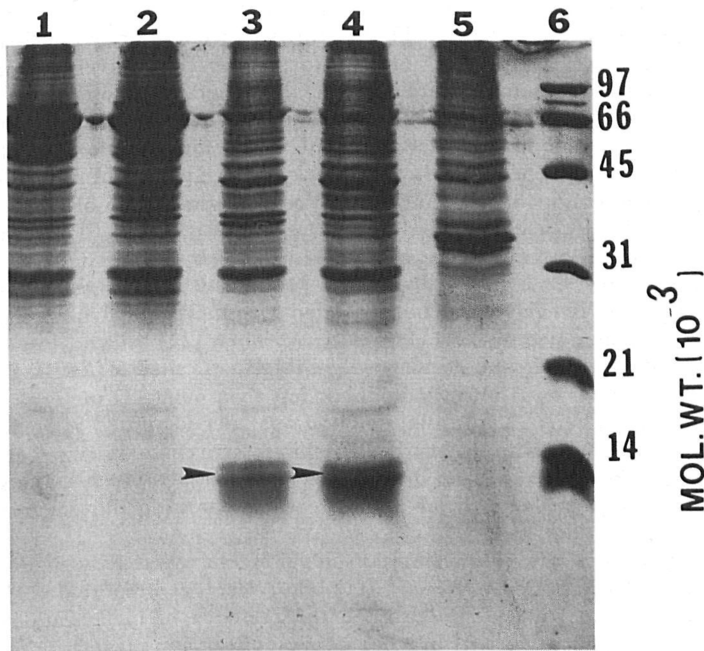

Figure 2. Presence of the 14-kD protein in post-grafted human skin extracts and supernatants. SDS-PAGE analysis was done on pre- and post-grafted human skin extracts and supernatants. Known concentrations (50 μg) of pre- and post-grafted skin extracts (2000 × *g*) and supernatants (100,000 × *g*) were subjected to SDS-PAGE (15% gel) under reducing conditions according to Laemmli's procedure [11]. The resolved proteins were stained with Coomassie blue (R-250). *Lane 1*, pre-grafted crude skin extract; *lane 2*, pre-grafted 100,000 × *g* skin supernatant; *lane 3*, post-grafted crude skin extract; *lane 4*, post-grafted 100,000 × *g* supernatant; *lane 5*, Triton X-100 supernatant of post-grafted skin; *lane 6*, low-molecular-weight markers. *Arrowheads* indicate migration of the 14-kD doublet, present exclusively in grafted skin extract (*lane 3*) and supernatant (*lane 4*).

resulted in no inhibition of either [^{3}H]thymidine or [^{3}H]proline incorporation (data not shown).

In Vivo Characterization of 14-kD Protein The injection of 14-kD band into 20 weeks post-grafted nude mice resulted in a marked reduction in the number of DOPA-positive melanocytes. The extent of reduction in melanocytes ranged from 9% to 32% **(Table II)**. Parallel to this decrease, skin color was also reduced at the site of injection. In contrast, no change in either DOPA-positive melanocytes or skin color was observed when the grafts were injected with normal saline. Microscopic analysis of histologic changes after injection of protein and/or normal saline was performed in a blinded manner. Based on hematoxylin and eosin

Table I. 14-kD Protein from Grafted Human Skin Inhibits Tyrosinase Activity in Normal Human Melanocytes[a]

	Tyrosinase Activity (cpm/10^6 cells)	Inhibition (% of Control)
Control	248,209 ± 20,619	0
Electroelution from donor skin		
0.25 μg/ml	217,698 ± 13,344	12.3 ± 5.3
0.50 μg/ml	214,315 ± 8,533	13.7 ± 3.4
Electroeluted protein from grafted skin		
0.25 μg/ml	156,909 ± 9,031	36.8 ± 3.6
0.50 μg/ml	116,677 ± 14,642	53.0 ± 5.9

[a] Data expressed as mean ± SD.

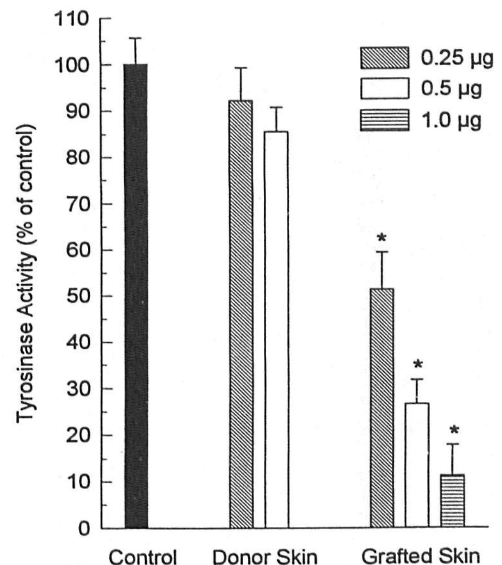

Figure 3. 14-kD protein from post-grafted skin inhibits tyrosinase activity in murine melanoma cells. Cells were inoculated at 0.2 × 10^6 cells/flask and were treated 48 h later with 0.25 to 1.0 μg/ml of 14-kD protein electroeluted from the gel. For control, an equal volume corresponding to 0.25 and 0.5 μg protein was added. *Diagonal-shaded bars* and *open bars* represent the volume corresponding to 0.25 and 0.5 μg/ml, respectively, of electroeluted protein from grafted human skin. The rest of the procedure was the same as described in the legend for **Fig 1**. Values are the mean of three determinations ± SD. * $p < 0.05$ (Student t test). Note a dose-dependent inhibition of tyrosinase activity in the presence of the 14-kD protein.

staining patterns, no difference between experimental and control animals was observed. There was no injury pattern in either the epidermis or dermis and no evidence of toxicity or inflammation. These results indicate that the electroeluted 14-kD protein when injected *in vivo* selectively inhibits melanocyte proliferation without causing histologic damage to epidermal cells.

To confirm further the inhibitory effect of the 14-kD protein on melanocyte proliferation *in vivo*, we studied human skin 2 weeks after grafting to athymic mice. These skin samples also showed a similar reduction in DOPA-positive melanocytes, which persisted throughout the experimental period.

DISCUSSION

The present study was performed to isolate and characterize the biologic factor(s) responsible for the observed hyperpigmentation. The stimulation of tyrosinase and cellular proliferation in these melanocyte cultures in the presence of extracts was monitored to assess the presence of stimulatory factor. An initial stimulation of tyrosinase activity and cell proliferation at lower concentration suggests the presence of a stimulatory factor, whereas at higher concentrations, inhibition of both tyrosinase activity and cellular proliferation indicates the presence of a melanogenic inhibitor in grafted skin.

Comparison of the protein profiles between pre- and post-grafted skin extracts revealed the presence of a doublet of approximately 14 kD exclusively in post-grafted skin. This protein has a potent inhibitory effect on pigmentation. Although a protein of similar molecular weight was also found in normal skin from nude mice, that protein had no effect on melanogenesis. Initial biochemical studies have indicated that the inhibitor is sensitive to protease treatment or ionic detergents, is thermolabile, and is completely inactivated at 100°C within 5 min. This is the first report of the presence of a potent melanogenic inhibitor in grafted human skin. This molecule is unique because it is a natural product of human skin. Initial *in vivo* studies of the isolated protein on early (2 weeks)

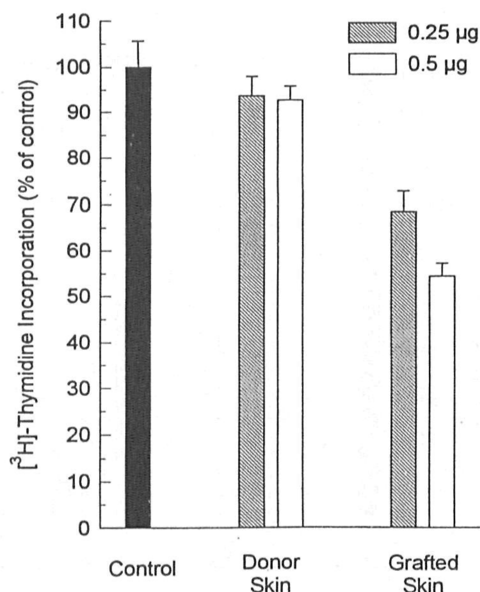

Figure 4. 14-kD protein inhibits cellular proliferation of murine melanoma cells. The cultures of murine melanoma cells in 96-well plates (4000 cells/well) were treated with 0.25 and 0.5 μg/ml of 14-kD protein electroeluted from post-grafted skin. For control, an equal volume corresponding to 0.25 and 0.5 μg protein was added. *Diagonal-shaded bars* and *open bars* represent the volume corresponding to 0.25 and 0.5 μg/ml, respectively, of electroeluted protein from grafted human skin. Three such treatments were given. Cell proliferation was measured in terms of [^{3}H]thymidine incorporation into total cellular DNA, as described in *Materials and Methods*. Values are the mean of three determinations ± SD. A concentration-dependent inhibition of [^{3}H]thymidine incorporation was noticed in cultures treated with 14-kD protein.

and late (20 weeks) grafted athymic mice suggested that the protein has a significant inhibitory effect, with no morphologic or anatomic changes to the grafted skin.

A number of reports have described the presence of melanogenic inhibitors [12–15]. At present, none of these inhibitors have been isolated or characterized rigorously. Recent studies by Kameyama *et al* [16] indicated the presence of a potent melanogenic inhibitor in nonpigmented murine JB/MS melanoma tumor cells. That inhibitor acts on tyrosinase to suppress melanin production dramatically. The identity and nature of that inhibitor has not yet been completely characterized. However, preliminary data suggest that unlike the melanogenic inhibitor reported here, that inhibitor is not sensitive to protease treatment or ionic detergents, but is inactivated by reducing agents. Recently, a post-tyrosinase inhibitor of melanogenesis, called blocking factor, has been described in S91 melanoma cells [17,18]. That factor blocks the conversion of 5,6-dihydroxyindole to indole 5,6 quinone and is lost after treatment of the cells with melanocyte-stimulating hormone. In another study, an inhibitor localized in the melanin granule fraction of S91 cells was described [19]. In addition, two large groups of melanogenic inhibitors have been documented. The first group of inhibitors did not affect isolated tyrosinase but was found to inhibit tyrosinase synthesis and its glycosylation, thereby suppressing melanogenesis in living cell systems [20]. The second group of inhibitors is effective in both isolated tyrosinase and living cell-suppression systems [21]. Recently, Mishima [22] isolated low–, middle–, and high–molecular-weight inhibitors called α (A&B), β, and γ, with α_1 A intrinsic inhibitor less than 6000 kD molecular weight. The α_1 A intrinsic inhibitor was later identified as lactic acid [22]. Lactic acid markedly inhibits tyrosinase isoenzymes, particularly T_2 and T_1 in B-16 cells, which suggests that it inhibits tyrosinase synthesis rather than directly suppressing the enzyme itself.

The presence of melanization-inhibiting factor activity in the ventral skin of an amphibian *Xenopus laevis* has been reported [23]. Subsequent studies demonstrated the presence of melanization-inhibiting factor activity in other species of frogs and suggested that this factor might play an important role in dorsal-ventral pigment pattern formation [24,25]. Unlike the inhibitor described here, that inhibitor has a molecular weight of approximately 450 kD. In addition to melanization-inhibiting factor, Fukuzawa *et al* reported a higher–molecular-weight inhibitor of approximately 1500 kD from frog skin, which inhibits severely both neural crest cell outgrowth and melanoblast differentiation.†

The identification of a melanogenic inhibitor in hyperpigmented skin grafts was unexpected. Rather, a potent melanogenic stimulator was anticipated. We speculate that this inhibitor may function as a feedback control in heavily melanized skin and that the relative ratios of melanogenic stimulators to inhibitors determines the ultimate melanogenic outcome: hyperpigmentation or hypopigmentation. If this is the case, then this would explain why inflammation results in either post-inflammatory hyper- or hypopigmentation. The melanogenic inhibitor that we have isolated might eventually be useful clinically for the treatment of various hyperpigmentary disorders.

† Fukuzawa T, Samaraweera P, Law JH, Bagnara JT: Preliminary characterization of an amphibian melanization inhibiting factor (abstr). *Pigment Cell Res* 2:451, 1989.

Table II. Multiple Injections of 14-kD Protein Inhibit DOPA-Positive Melanocytes in Grafted Nude Mice[a]

Group	Number of DOPA-Positive Melanocytes Before Injection	Injection of Electroeluted Protein		Injection of Normal Saline	
		Number of Melanocytes	% Reduction in Melanocytes	Number of Melanocytes	% Reduction in Melanocytes
A (experimental)					
92B (full ear)	635 ± 24	430 ± 31	32.3 ± 4.8	619 ± 6	2.5 ± 0.9
92A (both cut high)	690 ± 19	490 ± 15	29.0 ± 2.1	681 ± 5	1.3 ± 0.7
92A (full ear)	742 ± 30	559 ± 24	24.6 ± 3.3	740 ± 13	0
92A (right cut)	604 ± 30	550 ± 19	9.0 ± 3.1	600 ± 19	0
92A (left cut)	625 ± 27	561 ± 25	10.1 ± 4.0	613 ± 7	2.0 ± 1.1
B (control)					
92B (left cut)	586 ± 27	600 ± 21	0	600 ± 21	0
92B (both cut low)	502 ± 23	523 ± 18	0	523 ± 18	0

[a] Animals in group A were injected subcutaneously with 50 μl containing 2 μg of electroeluted proteins. Five such injections were given at weekly intervals on the left side of 20 weeks grafted mice. Similarly, the injections of normal saline were given on the right side of the graft in the same mice. Skin biopsies were taken before and after final injections, and DOPA-positive melanocytes were counted. In group B, no injections were given. Biopsies were taken before study and at 5 weeks to obtain baseline counts for DOPA-positive melanocytes. Data expressed as mean ± SD.

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