Transformation of Murine Melanocytes by Basic Fibroblast Growth Factor cDNA and Oncogenes and Selective Suppression of the Transformed Phenotype In a Reconstituted Cutaneous Environment

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Abstract. Constitutive expression of basic fibroblast growth factor (bFGF), a common characteristic of metastatic melanomas, was reproduced in vitro by infection of normal murine melanocytes with a recombinant retrovirus carrying a cDNA for bFGF. Expression of bFGF in these cells conferred autonomous growth in culture and extinguished differentiated functions, such as the synthesis of melanin and formation of dendrites. Independence from exogenous bFGF and loss of differentiated functions in vitro were induced also by transformation of melanocytes with the oncogenes *myc*, *Ela*, *ras*, and *neu*, although bFGF was not expressed by the respective transformants. As shown in skin reconstitution experiments onto syngeneic mice and subcutaneous injections into nude mice, the various transformants differed in their behavior in vivo. The bFGF transformants did not form tumors. They reverted to having a normal, melanotic phenotype and restricted growth. *Myc* and *Ela* transformants grew as tumors in nude mice but not in syngeneic, immunocompetent animals. *Ras*-transformed melanocytes were always tumorigenic, whereas the formation of tumors by *neu* transformants was suppressed by the concomitant grafting of keratinocytes in reconstituted skin of syngeneic mice. These data show that melanocytes genetically manipulated to produce bFGF acquire properties in vitro similar to those of metastatic melanoma cells or those induced by various oncogenes but that constitutive production of bFGF by itself is insufficient to make melanocytes tumorigenic. The experiments also show that melanocytes transformed by the selected oncogenes respond differentially to various environments in vivo.

INTERACTIONS among cells or tissues are mediated through direct cellular contacts or via the release of diffusable substances that act over a wide range of distances in minute quantities. An important question concerns the role of such interactions in the control of normal tissue homeostasis and the development of tumors. Skin is an ideal tissue in which to study these interactions, given the unique accessibility of its cells in vivo, in vitro, and upon reconstitution onto the animal.

Keratinocytes and fibroblasts are the major cell types in skin. Melanocytes make up a third population of cutaneous resident cells, with a well-defined course of differentiation and secretory function. They are important in photoprotection and are the cells of origin of one of the most malignant neoplasms, melanoma. Recent advances in the cultivation of melanocytes (Halaban, 1988) have defined the growth characteristics of these cells in vitro and allowed a comparison of some properties of normal melanocytes with those of their transformed counterparts. One of the most significant findings has been that normal melanocytes require exogenous basic fibroblast growth factor (bFGF)[1] to proliferate in culture (Halaban et al., 1987; Halaban, 1988). bFGF substitutes for the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) used routinely to grow mass cultures of normal melanocytes (Eisinger and Marko, 1982). In vivo, the requirement for exogenous bFGF appears to be met by basal keratinocytes and possibly dermal fibroblasts (Halaban et al., 1988*a*). bFGF is not produced by normal melanocytes but it is made constitutively by metastatic melanoma cells that grow in culture in the absence of added growth factor (Halaban et al., 1988*b*). Acquisition of the ability to produce bFGF may, therefore, be one of several transitional steps from a normal melanocyte to a highly malignant melanoma cell that is able to escape environmental control.

In this report, we evaluate the interplay between genetic events and cellular environment in control of melanocyte transformation and tumorigenesis. Murine melanocytes ex-

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1. *Abbreviations used in this paper*: bFGF, basic fibroblast growth factor; DOPA, L-β-3,4-dihydroxyphenylalanine; FGF, fibroblast growth factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

pressing bFGF or one of several activated oncogenes as a result of retroviral infection acquire a similar transformed phenotype in vitro but, as shown by skin reconstitution experiments, the similarities break down in vivo since the variously transformed melanocytes are affected differently by the cutaneous enviroment.

Materials and Methods

Cells

L-B10.BR melanocytes, established from newborn B10.BR mice (Tamura et al., 1987), were grown in Ham's F-10 medium (American Biorganics, Inc., N. Tonawanda, NY) supplemented with 48 nM TPA (Chemsyn Science Laboratories, Lenexa, KS), 10% newborn calf serum (Gibco Laboratories, Grand Island, NY), 200 U/ml penicillin, and 100 μg/ml streptomycin (Gibco Laboratories).

Primary keratinocytes were cultured from newborn B10.BR mice as described by Yuspa and Harris (1974) and plated (10^7 cells/55-cm^2 dish) in MEM (Gibco Laboratories) at low $CaCl_2$ concentration (0.05 mM) supplemented with 4% Chelex-treated FBS (Flow Laboratories, McLean, VA) and 10 ng/ml epidermal growth factor (Collaborative Research, Bedford, MA) as previously described (Hennings et al., 1980; Dotto et al., 1986, 1988). Keratinocyte cultures were used for grafting 7–10 d after plating. Under these conditions, the primary keratinocyte cultures were free of contaminating fibroblasts but retained some melanocytes, which were carried over at the time of grafting.

Dermal fibroblasts were prepared from newborn B10.BR mice as described (Dotto et al., 1988). Cells were plated (2×10^6 cells/55-cm^2 dish) in DME (Gibco Laboratories) supplemented with 10% calf serum (Gibco Laboratories). Cells were grown to confluence (in 1–2 d) and split (1:4) twice before grafting.

Retroviral Vectors

The MD-bFGF virus was constructed by inserting a complete 1.4-kb cDNA copy of bovine fibroblast growth factor (FGF) (Abraham et al., 1986) behind the internal SV-40 promoter of the retroviral vector MD at its unique Bgl II site (Fig. 1). Details of the construction of the MD vector will be presented elsewhere. Briefly, the vector was derived from pC663, a plasmid clone containing the complete genome of myeloproliferative sarcoma virus (Stocking et al., 1985). A gene for resistance to G418 (Southern and Berg, 1982) was inserted at the unique Bam HI site of pC663, while the internal part of the viral genome (from the unique Bam HI to Cla I sites) was replaced with a cassette containing the SV-40 early promoter (Korman et al., 1987) and, behind it, a unique cloning site for either Bgl II/Bam HI or Xho I/Sal I. *Psi*-2 helper cells (Mann et al., 1983) were transfected with the MD-*bFGF* recombinant construct, and one clone was selected for production of high-viral titers (measured in terms of G418-resistant colony forming units per milliliter).

The *ras*-zip 6 and VM-*myc* retroviruses carry the viral Harvey *ras* and avian MC29 *gag-myc* oncogenes, respectively (Dotto et al., 1985).

The Glu664-*neu* virus carries a complete cDNA copy of the transforming rat *neu* oncogene as described by Bargmann and Weinberg (1988). This virus is able to induce full transformation of established fibroblasts (Bargmann and Weinberg, 1988) and keratinocytes (Dotto et al., 1989) with concomitant high expression of the *neu* oncoprotein.

The Mp-*p53* virus carries a complete cDNA copy of a *p53* gene of murine origin (Wolf et al., 1985) inserted into a derivative of the DOL vector (Korman et al., 1987) where the MuLV long terminal repeat of the vector had been substituted with a long terminal repeat derived from MpSV (Stocking et al., 1985). Biological activity of this virus was demonstrated in a previous study by transformation of papilloma-derived keratinocytes (Dotto et al., 1989). MD-*p53* is essentially equivalent to Mp-*p53* except that it was constructed by insertion of the *p53* cDNA copy into the MD vector at the Bgl II site.

The MD-*Ela* virus contains a cDNA copy of the *13s* transcript of the *Ela* region of adenovirus 5 (Roberts et al., 1985) inserted behind the SV-40 promoter of the MD vector. High-titer *psi*-2 producer cells (Mann et al., 1983) were used for subsequent studies. Infection of fibroblasts with this virus conferred high expression of the *Ela* gene product and readily caused morphological transformation of monolayer cultures, inducing a typical cobblestone appearance (Roberts et al., 1985).

The pC6M-*neo* virus (Stocking et al., 1985) carries only the G418 resistance gene with no oncogene and was used as a negative control.

Infection of Melanocytes and Selection of Transformants

Melanocyte cultures, at ~25% confluency in 25-cm^2 flasks, were infected with the various retroviruses by a 2-h exposure to undiluted viral stock with the addition of 8 μg/ml polybrene (Aldrich Chemical Co., Milwaukee, WI). The viral preparations were then removed and replaced with melanocyte medium supplemented with TPA. Infected cultures were grown to confluency (7–10 d) and then split (1:2) into selective media containing 800 μg/ml G418 (Gibco Laboratories) with or without TPA.

In Vitro Characterization of Transformed Melanocytes

Growth. Kinetics of cell proliferation with and without G418, with and without TPA, were measured by seeding melanocytes in 12-well plates (~10^4 cells/cm^2) and counting cells from two wells in a cell counter (Coulter Electronics Inc., Hialeah, FL) at intervals of 2–3 d. Population doubling times were calculated from the slopes of the linear portions of the growth curves.

Mitogenicity. Assays for the mitogenic activity present in cell extracts, described by Halaban et al. (1987), involved addition of crude extracts made from the various transformants to cultures of normal human melanocytes (in duplicate 2-cm^2 well, 24-well cluster plates) in chemically defined PC-1 medium (Ventrex Laboratories, Inc., Portland, ME) plus 1 mM dibutyryl cAMP (Sigma Chemical Co., St. Louis, MO) for 24 h. During the last 3 h, the cells were incubated in MEM without calcium and magnesium (Gibco Laboratories) supplemented with 5 μCi/ml [^{3}H]thymidine (90 Ci/mmole; Amersham Corp., Arlington Heights, IL). The cells were then detached from the culture dishes with trypsin-EDTA solution and trapped onto No. 30 glass filters in the minifold apparatus of Schleicher & Schuell, Inc. (Keene, NH). The filters were washed three times with distilled water, dried, and placed in scintillation fluid, and radioactivity was determined in a scintillation counter.

bFGF and Oncogene Expression

Immunoprecipitation. Specific gene products were immunoprecipitated after metabolic labeling of cells with 250 μCi/ml [^{35}S]methionine (for bFGF and *p53* transformants) or [^{35}S]cysteine (for *neu* transformants) in methionine- or cysteine-free DME for 0.5 or 14 h, respectively. Immunoprecipitations with anti-bFGF-(1–24) (Halaban et al., 1987), anti-*p53*, and anti-*neu* antibodies were performed as described (Halaban et al., 1983). The PAb421 monoclonal antibodies against *p53* (Harlow et al., 1981) were a gift of Dr. A. Levine (Department of Biology, Princeton University, Princeton, NJ). The monoclonal antibody 7.16.4 against the *neu* gene product (Drebin et al., 1984) was provided by Dr. D. F. Stern (Department of Pathology, Yale University, New Haven, CT). Immunoprecipitated proteins were resolved by SDS-PAGE, and dried gels were fluorographed.

Immunoblotting. Total cell extracts, prepared in electrophoresis sample buffer (5 mM sodium phosphate, pH 7, 2% SDS, 0.1 M DTT, 5% 2-mercaptoethanol, 10% glycerol, 0.4% bromophenol blue) were run on discontinuous 10% acrylamide, 0.13% bisacrylamide gels. The gels were washed for 5 min in water and once in transfer buffer, and proteins were transferred to nitrocellulose by electrophoresis for 2 h at 50 V in the cold. The transfer buffer and procedures for blocking and washing the filters were as described by Kamps and Sefton (1988). Blots were incubated with monoclonal antibody Ab-1 against adenovirus *Ela* gene product (Oncogene Science, Inc., Manhasset, NY) followed by incubation in blocking buffer containing 1 μCi/ml ^{125}I-protein A (ICN Radiochemicals, Costa Mesa, CA; 35 μCi/μg) and placed on film for autoradiography.

RNA Blotting. 20 μg of total cellular RNA was run on an agarose gel and blotted as described by Dotto et al. (1989). Probes were prepared by gel purification of DNA fragments corresponding to v-Harvey *ras* and MC29 *myc* oncogenes (Dotto et al., 1985) and labeled with [^{32}P]dCTP by random oligonucleotide priming as described by Feinberg and Vogelstein (1983).

Grafting

Cells were grafted by the technique of Worst et al. (1982) as described (Dotto et al., 1988, 1989). At first, a glass disk was implanted subcutaneously onto the back of each mouse. 2–4 wk later, the glass disk was replaced

by a dome-shaped, open-bottom silicon chamber, which provided a barrier to invasion of the graft bed by the surrounding host skin. Cells were then suspended in MEM (Gibco Laboratories) at low calcium concentration (0.05 mM), mixed in various combinations, and injected into the chambers. They readily attached to the underlying granulation tissue and proceeded to form stratified skin in 2–3 wk. The numbers used for injection of each cell type into the chambers were 2×10^5 melanocytes (normal or transformed), 2×10^6 keratinocytes, and 8×10^6 dermal fibroblasts. Mice were killed 21–28 d later, and the graft tissues were fixed for histologic and electron microscopic analysis.

Electron Microscopy

Tissues retrieved from the grafts were immersed in cold aldehyde and sliced vertically to allow embedding in an oriented fashion. Cells in culture were trypsinized, fixed as pellets, and processed like a piece of tissue. Further treatments, including histochemical incubation with DOPA for the localization of tyrosinase, were described elsewhere (Halaban et al., 1988*c*; Slominski et al., 1988).

Results

Transformation of Melanocytes In Vitro by bFGF cDNA

A recombinant retrovirus (MD-bFGF; Fig. 1) carrying a complete cDNA copy of bovine bFGF (Abraham et al., 1986) was constructed starting from the retroviral vector MD. Production of a bFGF-like factor by NIH-3T3 cells infected with this virus was verified by a mitogenicity assay with human melanocytes as test cells (Table I).

MD-bFGF was used to infect melanocytes of the L-B10.BR cell line (Tamura et al., 1987). Although these cells have been passaged in culture for a relatively long time and have lost some of the characteristics of primary melanocytes, such as dependence on external inducers of cAMP, they have retained many other normal properties, including pigment forming ability, TPA and bFGF dependence in culture, and inability to form tumors in animals (our unpublished observations). The chromosome complement of these cells is close to normal, diverging only by inclusion of an isochromosome 6 (Tamura et al., 1987).

Approximately 10% of L-B10.BR melanocytes from cultures infected with the MD-bFGF virus grew in the presence of G418, and a subfraction acquired the ability to grow in the absence of TPA (Fig. 2). Independence of the melanocytes from TPA after MD-bFGF infection was associated with loss of melanocyte-specific functions, such as tyrosinase activity, melanogenesis (Table II and Fig. 3), and the ability to form

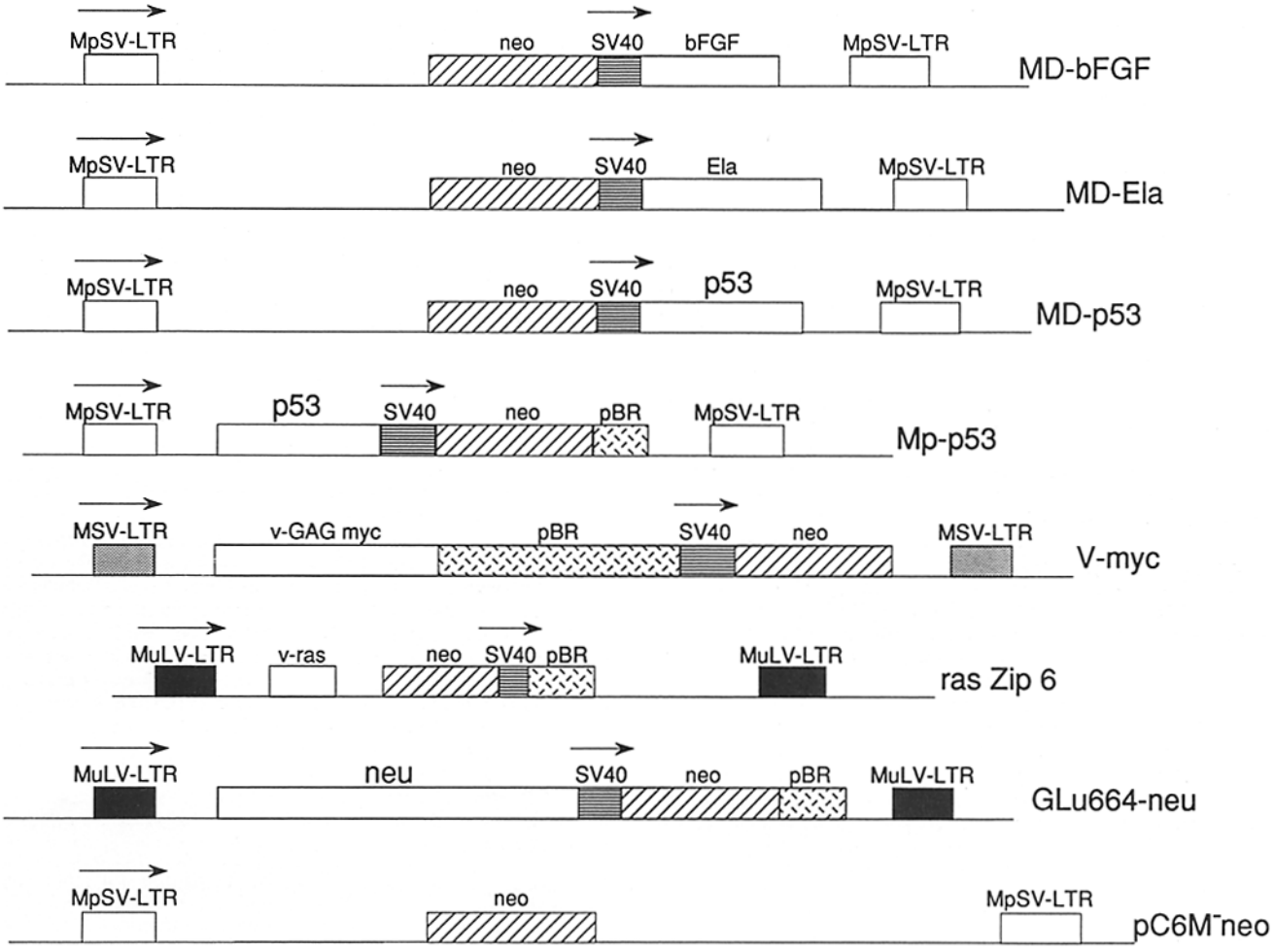

Figure 1. Schematic diagram of the retroviral constructs used in these studies. The viruses are described in Materials and Methods. Indicated are the transduced genes, included the G418 resistance gene (*neo*), as well as the direction of the transcription from the SV-40 promoter and from the long terminal repeats derived from myeloproliferative sarcoma virus (*MpSV-LTR, clear boxes*), murine sarcoma virus (*MSV, stippled boxes*), and Moloney leukemia virus (*MuLV, solid boxes*).

Table I. Mitogenic Activity of Cell Extracts

	Additions.	[^{3}H]Thymidine incorporation by human melanocytes
		cpm/well per 3 h
Experiment 1	bFGF	14,750
	NIH-3T3 fibroblast extract	1,560
	NIH-3T3-*bFGF* fibroblast extract	17,220
	No additions	270
Experiment 2	bFGF	38,500
	L-B10.BR-*bFGF* melanocyte extract	12,500
	Extracts from L-B10.BR melanocytes transformed with other viruses	<420
	Conditioned medium from L-B10.BR-*bFGF* and NIH-3T3-*bFGF* transformants	<180
	No additions	<610

Human melanocytes were seeded in PC-1 medium plus 1 mM dibutyryl cAMP in 24-well cluster plates (~40,000 cells/well) with and without the various additions. [^{3}H]Thymidine was incorporated during the last 3 h of a 24-h incubation. Additions were 1 ng/ml bFGF (Collaborative Research, Inc.); extracts from NIH-3T3 and NIH-3T3-*bFGF* cells at 10 μg ml; and L-B10.BR melanocytes transformed with bFGF cDNA or *myc*, *neu*, *ras* or *E1a* oncogenes at 50 μg protein/ml. Values are averages of counts per minute from two wells. Pure bFGF or cell extracts without dibutyryl cAMP were not mitogenic toward human melanocytes (Halaban et al., 1987; data not shown).

dendritic cell processes (Fig. 3). At passage 20, no premelanosomes were found by electron microscopy, and, despite the presence of an elaborate Golgi apparatus, the *trans*-Golgi reticulum was negative for DOPA oxidase (tyrosinase) activity (Fig. 4).

Extracts of transformed L-B10.BR melanocytes were tested in the mitogenicity bioassay with human melanocytes. Mitogenic activity toward normal melanocytes was present in extracts of MD-bFGF-transformed melanocytes (Table I) but not in extracts prepared from uninfected cells (data not shown) or cells infected with known oncogenes (Table I). The mitogenic agent was not detectable in the culture medium of MD-bFGF-transformed melanocytes (Table I) as had been shown also for human melanoma cells and normal human keratinocytes, both of which when used as cell extracts were mitogenic toward normal human melanocytes (Halaban et al., 1988*a*,*b*). bFGF in MD-bFGF-transformed melanocytes was demonstrated by immunoprecipitation with anti-bFGF antibodies (Fig. 5).

L-B10.BR melanocytes infected with the control pC6M-*neo* virus (Stocking et al., 1985), which transduces only the G418 resistance gene (Fig. 1), were able to proliferate in the presence of G418 but remained dependent on TPA for growth (data not shown). No colonies of proliferating cells appeared upon removal of TPA, even after 3 mo of cultivation in the presence of TPA and G418, a time span corresponding approximately to eight population doublings. These melanocytes retained their dendritic shape and their ability to synthesize melanin (Fig. 3) and tyrosinase (Table II). In the absence of TPA, the cells flattened out in a manner characteristic of growth-arrested melanocytes (Fig. 3).

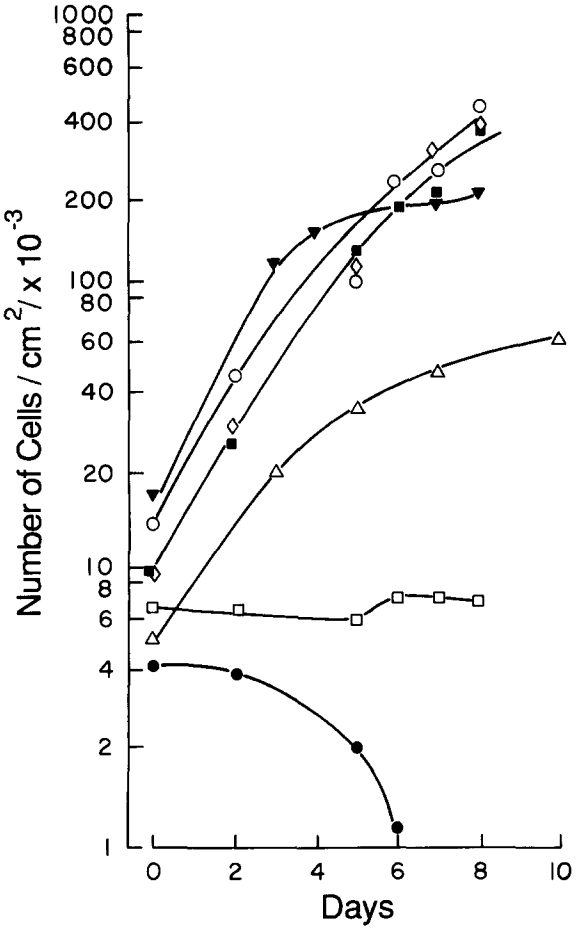

Figure 2. Growth kinetics of control and variously transformed melanocytes in the absence of TPA. Melanocytes were plated in medium containing G418 (800 μg/ml). Included in this experiment were uninfected (control) L-B10.BR melanocytes (●) and melanocytes infected with viruses MD-*p53* (□), MD-bFGF (△), VM-*myc* (▼), MD-*Ela* (■), *ras*-zip 6 (◇), and Glu664-*neu* (○).

In Vitro Transformation of Melanocytes by Known Oncogenes

Several oncogenes encoding nuclear, cytoplasmic, or mem-

Table II. Expression of Differentiated Functions In Vitro

Melanocyte culture	Tyrosinase activity	Pigmentation
	μU/mg protein	
LB10.BR	250–1,800	+++
L-B10.BR-*neo*	510	+++
L-B10.BR-*p53*	1,640	+++
L-B10.BR-*bFGF*	13	–
L-B10.BR-*myc*	2	–
L-B10.BR-*Ela*	2	–
L-B10.BR-*ras*	7	–
L-B10.BR-*neu*	1	–

Tyrosinase assays were performed as described by Halaban et al. (1983) with extracts from the various types of melanocytes 1.5–3 mo after selection for growth in the absence of TPA but in the presence of 800 μg/ml G418-sulfate. A unit of tyrosinase was defined as the activity of enzyme that catalyzed the oxidation of 1 μmol of tyrosine/min. Assays were performed for 10–30 min. Melanocytes that lost their pigmentation could not be stimulated to regain it by the addition of TPA and/or isobutylmethyl xanthine. Pigmentation was assessed by the color of the cell pellets (see Fig. 3).

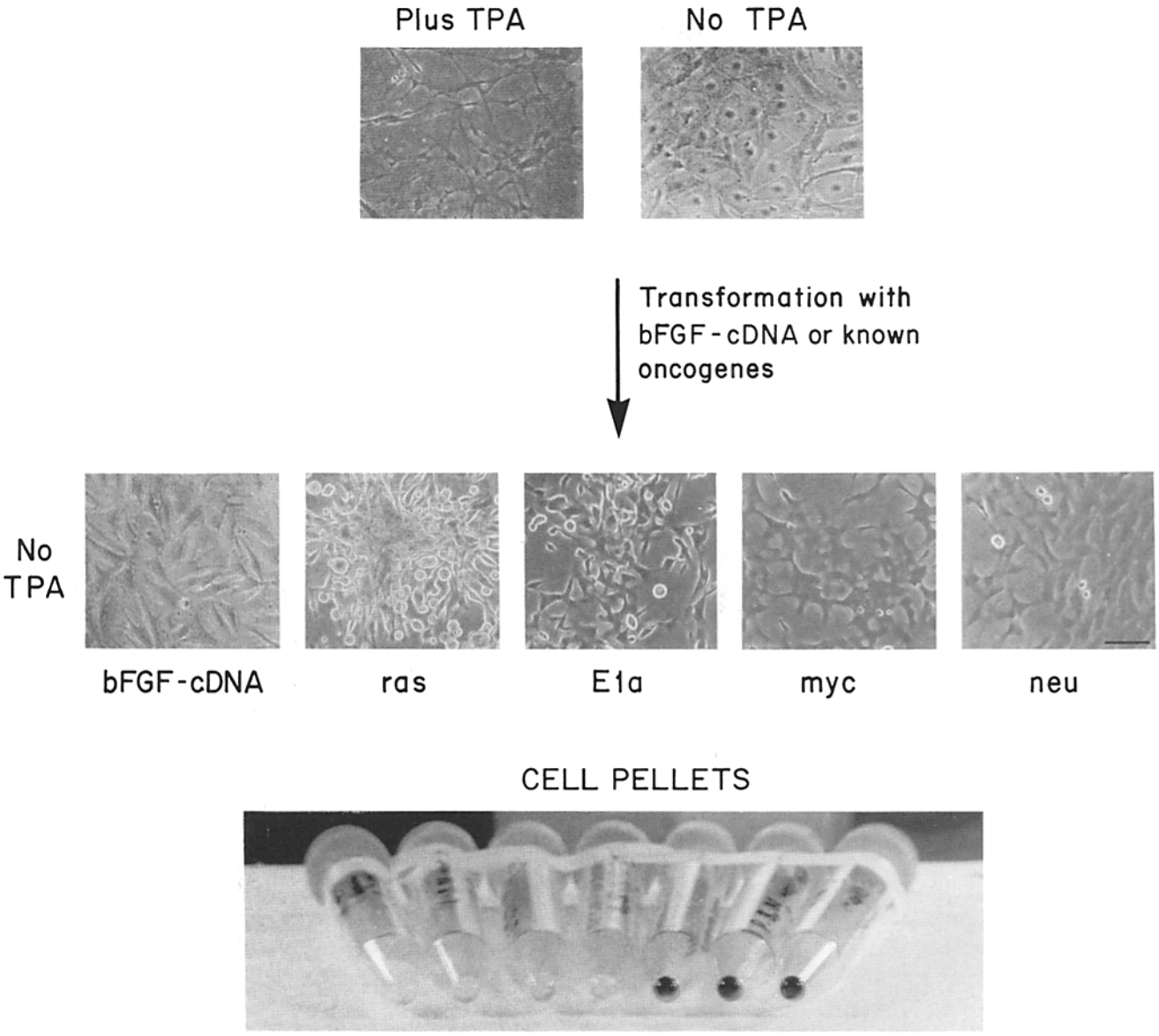

Figure 3. Phase–contrast micrographs of untransformed and transformed melanocytes in culture and loss of pigmentation as demonstrated in cell pellets. (*Top*) Untransformed L-B10.BR in the presence of TPA or 24 h after withdrawal of TPA. (*Middle*) Transformed melanocytes growing in medium lacking TPA. Morphology of cells infected with the pC6M$^-$*neo*, MD-*p53*, and Mp-*p53* was indistinguishable from that of untransformed melanocytes in the absence of TPA (*Top, right*). (*Bottom*) Amelanotic *bFGF, ras, neu,* and *Ela* transformants are shown from left to right followed by melanotic pC6M$^-$*neo*– and MD-*p53*–infected cells and parental L-B10.BR melanocytes. Bar, 17 μm.

brane-bound gene products were introduced into L-B10.BR melanocytes by means of retroviral vector infection (Fig. 1). As above, cells were selected for acquisition of G418 resistance and tested for their ability to grow in medium not supplemented with TPA.

Of three oncogenes encoding nuclear proteins—the mutated, oncogenic form of murine *p53* (Wolf et al., 1985; Finlay et al., 1988), avian v-*myc* (Dotto et al., 1985), and adenovirus *Ela* (Roberts et al., 1985)—only v-*myc* and *Ela* were able to transform melanocytes. L-B10.BR cells infected with either of two *p53* recombinant viruses, Mp-*p53* or MD-*p53* (Fig. 1), remained indistinguishable in phenotype from their uninfected or pC6$^-$*neo*–infected counterparts (Figs. 2 and 3) despite the expression of viral oncoprotein as shown by immunoprecipitation with anti-*p53* monoclonal antibody (Fig. 5).

L-B10.BR melanocytes infected with a retroviral vector carrying the avian MC29 *gag-myc* oncogene, VM-*myc* (Dotto et al., 1985), initially behaved like Mp- or MD-*p53*–infected cells: whereas little or no proliferation occurred in the absence of TPA, many cells acquired the ability to grow in the presence of G418 in permissive (with TPA) medium and remained pigmented and dendritic. After 3–4 wk of TPA starvation, however, colonies of cells appeared that were able to grow in a restrictive (without TPA) medium (Fig. 2). Like the cells transformed by MD-bFGF, the TPA-independent v-*myc* transformants were amelanotic, with extremely low tyrosinase activity (Table II and Fig. 3). Ability

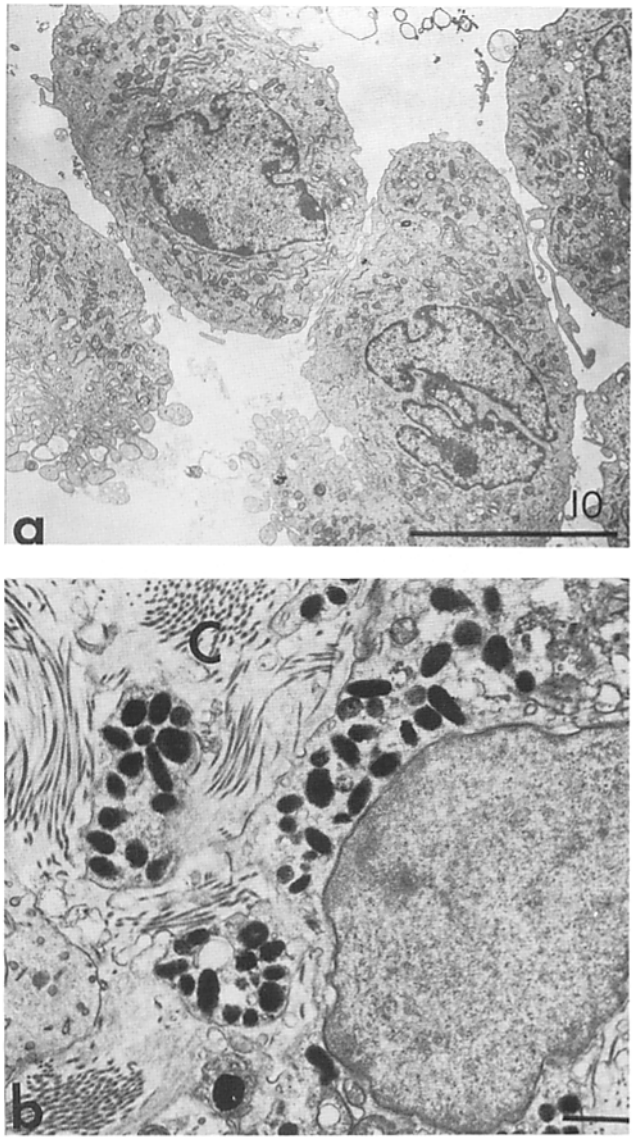

Figure 4. Electron micrographs of MD-bFGF–transformed L-B10.BR melanocytes in vitro and in the dermis of incompletely reconstituted syngeneic skin. Pure populations of *bFGF* transformants were grafted without addition of keratinocytes or dermal fibroblasts. Illustrated are the loss of melanogenesis and DOPA reactivity in vitro (*a*) and reversion to the melanotic phenotype in the dermal environment in vivo (*b*). *C*, collagen bundles. Bars: (*a*) 10 μm; (*b*) 1 μm.

to grow in the absence of TPA correlated with elevated levels of v-*myc*-RNA expression (Fig. 5).

L-B10.BR melanocytes were transformed rapidly by infection with a virus carrying a third "nuclear" oncogene, the adenovirus *Ela-13s* region (Roberts et al., 1985) (MD-*Ela*; Fig. 1). 3–4 d after infection with MD-*Ela*, the great majority of melanocytes acquired the ability to proliferate equally well whether or not TPA was present (Fig. 2). Rapid proliferation was accompanied by loss of pigmentation and change in morphology, which, in turn, correlated with a drastic decrease in tyrosinase activity (Table II and Fig. 3). MD-*Ela*–infected L-B10.BR melanocytes expressed high levels of the *Ela* gene product as shown by immunoblotting with *Ela*-specific monoclonal antibodies (Fig. 5).

Melanocytes infected with viruses carrying the v-Harvey *ras* or rat *neu* oncogenes (*ras*-zip 6 and Glu664-*neu*; Fig. 1), encoding, respectively, a cytoplasmic and a plasmalemmal protein (Dotto et al., 1985; Bargmann and Weinberg, 1988), were transformed with the same rapidity as those infected with the MD-*Ela* virus and were, like the latter, distinguishable in the phase microscope by their altered morphology within 3–4 d (Fig. 3). The cells grew rapidly under all culture conditions tested (Fig. 2) and quickly lost their pigment-forming ability (Table II and Fig. 3). Oncogene expression in the transformants was verified by RNA blotting (*ras*) or immunoprecipitation (*neu*) (Fig. 5).

The amelanotic *ras* and *neu* transformants were examined with the electron microscope at passages 15 and 17, respectively, and were found to contain residual melanosomes. These were segregated in large compartments presumed to be autophagosomes (*ras* transformants) or they were dispersed singly (*neu* transformants; Fig. 9 *a*, *insert*). Cultures from both transformants included cells that, on incubation with DOPA, showed evidence of tyrosinase in elements of the *trans*-Golgi reticulum. Many cells contained DOPA-positive lysosome-like granules, similar to the "granular melanosomes" observed in cultures of amelanotic Bomirski hamster melanoma cells after induction of melanogenesis by L-tyrosine (Slominski et al., 1988).

Tumorigenicity Assay I: Skin Reconstitution Experiments

An important criterion for cell transformation involves assessment of tumorigenic behavior. In previous work with oncogene-transformed keratinocytes, skin reconstitution experiments provided a sensitive tumorigenicity assay of malignant transformation at stages not easily detected by conventional methods (Dotto et al., 1988, 1989). In addition, and perhaps more importantly, these grafting assays allowed a study of the effects of various cellular permutations under conditions that approximate a normal cutaneous environment (Dotto et al., 1988).

Grafting experiments onto syngeneic B10.BR mice were performed by use of silicon transplantation chambers into which cultured skin cells were injected in various combinations. Grafting of normal L-B10.BR melanocytes together with primary keratinocytes and dermal fibroblasts resulted, 3–4 wk later, in quasinormal skin, with appropriate reassortment of cells (Fig. 6). The transferred melanocytes functioned normally (Fig. 7) and were located within or immediately beneath the newly formed epidermis (in cases where only keratinocytes and melanocytes were grafted) or, in addition, admixed with the dermal fibroblasts (when the latter were grafted also). As expected, tumors were not formed by untransformed L-B10.BR melanocytes (Table III).

Despite their transformed phenotype in vitro, melanocytes carrying the MD-bFGF virus were not tumorigenic (Table III). These highly proliferative, growth factor–independent, amelanotic cells (Figs. 2 and 3) behaved after grafting like their parental cell line (Fig. 6) and resumed production of pigment (Figs. 4 and 6). Reversion to this normal phenotype did not depend on concomitant grafting of fibroblasts and/or epidermal cells (Table III).

Similar lack of tumorigenicity was observed after grafting of *myc* or *Ela* transformants, both in the presence and absence of keratinocytes and dermal fibroblasts (Table III).

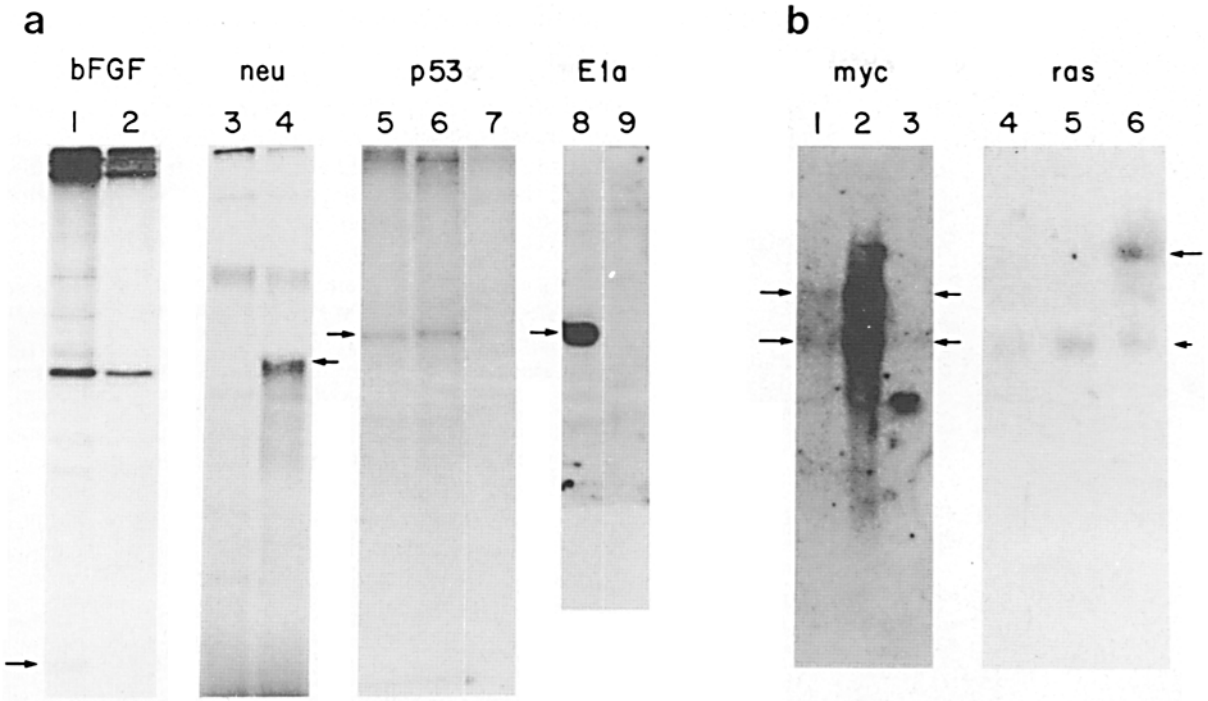

Figure 5. Expression of virally transduced genes. (*a*) Protein expression was detected by immunoprecipitation (*bFGF, neu,* and *p53*) or immunoblotting (*Ela*) with corresponding antibodies as described in Materials and Methods. (Lane *1*) bFGF-transformed melanocytes; (lane *4*) *neu* transformants; (lanes *5* and *6*) cells infected with MD-*p53* and Mp-*p53* viruses, respectively; (lane *8*) *Ela* transformants; and (lanes *2, 3, 7,* and *9*) negative controls with normal L-B10.BR cells. Arrows indicate proteins of the expected molecular mass: 17, 185, 53, and 50 kD for bFGF, *neu*, *p53*, and *Ela*, respectively. (*b*) Oncogene-specific mRNA expression was detected by Northern blotting with DNA probes for v-*myc* (lanes *1–3*) and V-Ha-*ras* (lanes *4–6*) as described in Materials and Methods. (Lane *1* and *2*) VM-*myc*–infected cells selected for G418 resistance only or for G418 resistance and bFGF independence, respectively; (lane *3*) uninfected control melanocytes; (lane *6*) *ras*-zip6–infected melanocytes; (lane *4*) uninfected cells; (lane *5*) cells infected with the Glu664-*neu* virus and used here also as a negative control. In the case of VM-*myc*–infected cells (lanes *1* and *2*), two specific bands were detected of which one is likely to correspond to the full-length viral transcript (~6 kb). The other is a small segment that might be a product of internal splicing. The intensity of both bands is markedly higher in the bFGF-independent transformants than in their bFGF-dependent counterparts. In the case of *ras*-zip6–infected cells (lane *6*), two bands were again detected, of which one corresponds to the full-length viral transcript (~5 kb) and the other represents the endogenous c-Ha-*ras* transcript (~1.2 kb), detectable also in the uninfected controls (lanes *4* and *5*). Approximate molecular weights were calculated from the migration of ribosomal RNA marker bands (not shown).

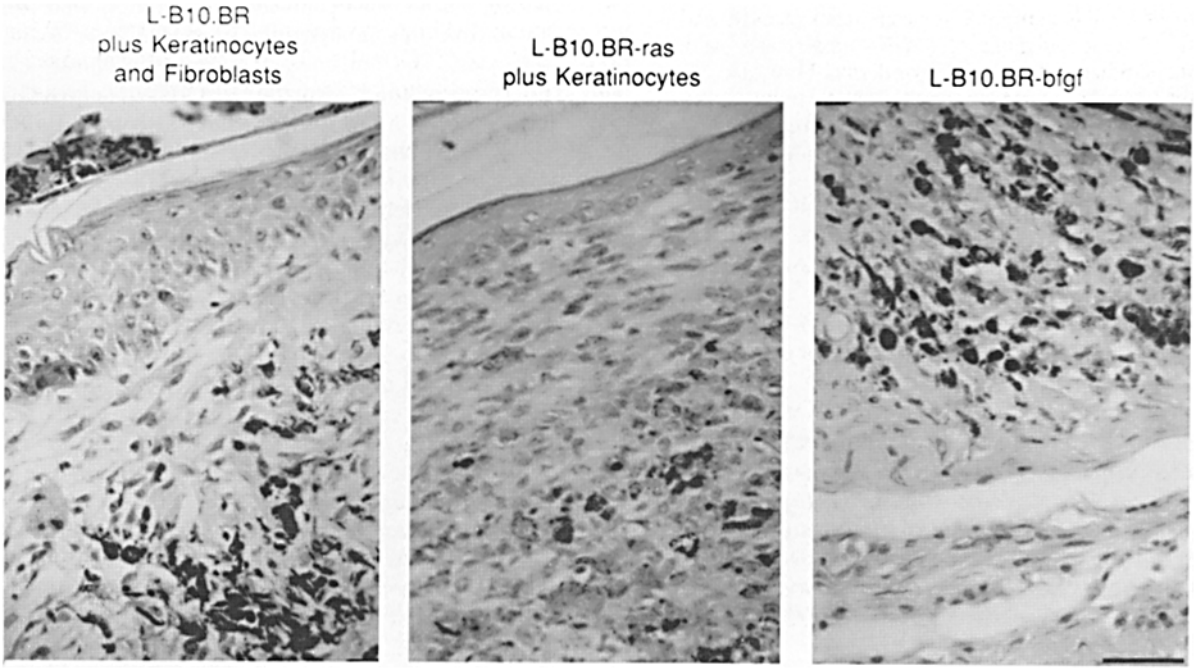

Figure 6. Histology of grafts in partially or fully reconstituted syngeneic skin containing control melanocytes or *ras* or *bFGF* transformants. Shown are untransformed L-B10.BR melanocytes grafted with keratinocytes and dermal fibroblasts, *ras*-transformed melanocytes grafted with normal keratinocytes, and *bFGF* transformants grafted as a pure cell population without keratinocytes or dermal fibroblasts. Mice were killed, and tissues were fixed 4 w after grafting. Bar, 23 μm.

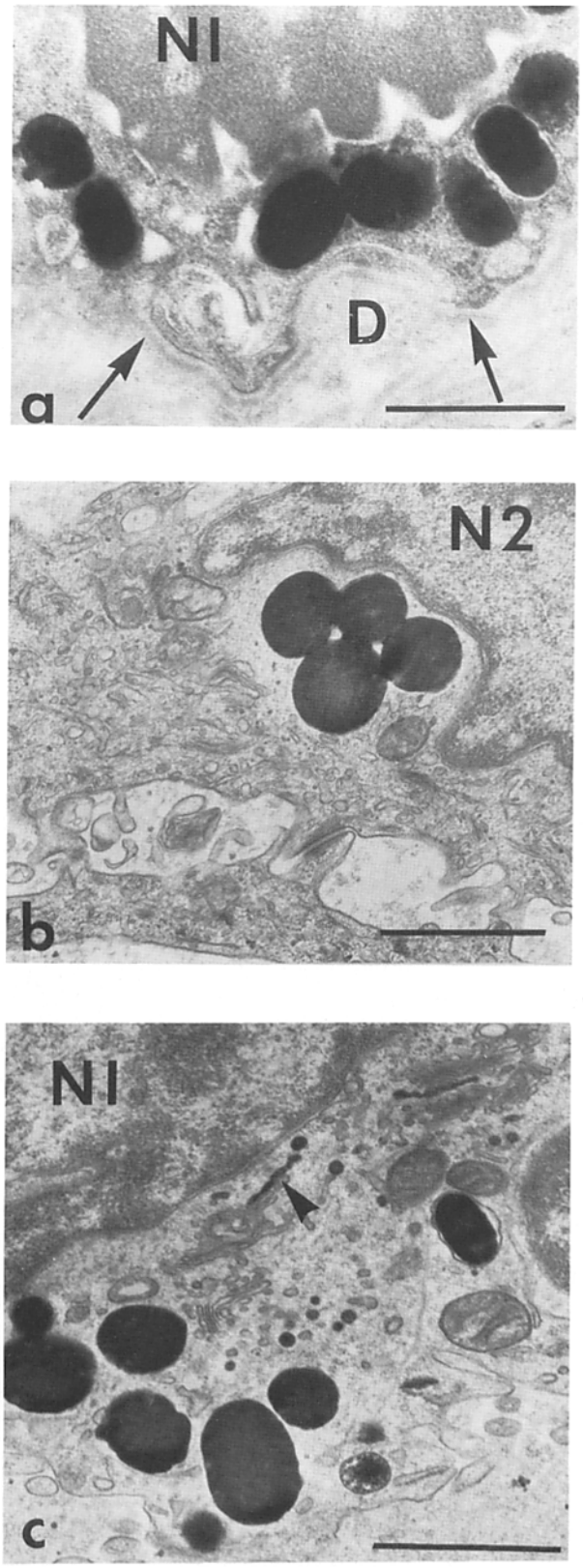

Figure 7. Normal function of melanocytes in fully reconstituted syngeneic skin as detected by electron microscopy. Sorting of cells to form a new epidermis from an injected suspension of untransformed L-B10.BR melanocytes, keratinocytes, and dermal fibroblasts resulted in normal basal location of melanocytes (*a*), transfer of melanin granules to keratinocytes (*b*), and active melanogenesis (*a* and *c*). The large electron-dense granules are fully melanized melanosomes, and the small ones transport vesicles of tyrosinase, visualized with DOPA. *NI*, nucleus of melanocyte; *D*, dermis; *N2*, nucelus of keratinocyte. The arrows indicate basement membrane, and the arrowhead indicates histochemical DOPA reaction product showing presence of tyrosinase in *trans*-Golgi reticulum. Bars 1 μm.

Table III. Tumor Growth in Reconstituted Skin

Cells	M	M+K	M+F	M+K+F
L-B10.BR	ND	0[0]:3	ND	0[0]:5
L-B10.BR-*bFGF*	0[0]:7	0[0]:3	ND	ND
LB10.BR-*ras*	3[4]:4	7[7]:7	1[1]:1	7[7]:7
LB10.BR-*neu*	4[4]:8	0[1]:8	6[6]:6	1[3]:10
LB10.BR-*myc*	0[1]:4	ND	ND	0[2]:3
LB10.BR-*E1a*	0[1]:2	ND	ND	0[2]:3

Normal or variously transformed L-B10.BR melanocytes (*M*) were grafted onto syngeneic B10.BR mice either alone or in association with primary keratinocytes (*K*) and/or dermal fibroblasts (*F*). Number of cells injected and grafting procedure were described in Materials and Methods. Mice were killed 21–28 d after grafting, and the graft tissue was fixed in formalin for histologic analysis. The ratio of macroscopically detectable tumors to total number of grafts performed with a certain combination of cells is given. Between brackets is the number of histologically positive tumors. In all cases, data were pooled from several independent experiments.

However, histologic analysis revealed some foci of limited neoplastic proliferation containing a few weakly pigmented cells (Table III and data not shown).

Melanocytes transformed with *ras* were tumorigenic. Small, mostly exophytic and unpigmented tumors formed consistently, irrespective of the presence or absence of dermal fibroblasts and/or keratinocytes (Table III). Spindle-shaped and large cuboidal cells infiltrated surrounding tissue. Gray portions of the tumors contained pigmented cells with a foamy appearance, suggesting that these might be macrophages that had ingested melanin (Fig. 6). Concomitant grafting of keratinocytes resulted in formation of a stratified epidermis on top of the tumors (Fig. 6). No metastatic spread was detected to liver, lung, or spleen over the duration of the experiments (3–4 wk).

Similar to the *ras* transformants, *neu*-transformed melanocytes were able to grow into amelanotic tumors (Fig. 8, *top*, and Fig. 9 *a*) when grafted alone or with dermal fibroblasts (Table III). Dermal fibroblasts enhanced the growth of these tumors (Table III). In contrast, when *neu*-transformed melanocytes were grafted with keratinocytes, tumor formation was drastically suppressed (Fig. 8, *bottom*; Fig. 9 *b*; Table III). Microscopic foci of neoplastic proliferation were detected in one case only in areas that happened to be devoid of keratinocytes. The presence of dermal fibroblasts enhanced microscopic tumor formation to a limited degree (Table III). In all cases, a few differentiated melanocytes were present underneath the newly formed epidermis, some in close apposition to lymphocytes (Fig. 9 *b*). These might be phenotypically reverted *neu* transformants or normal melanocytes carried over with the primary keratinocyte cultures.

Tumorigenicity Assay II: Subcutaneous Injection of Transformants into Nude Mice

The grafting experiments described above made use of syngeneic, immunocompetent mice. To explore a possible role of the immune system in modulating the behavior of the vari-

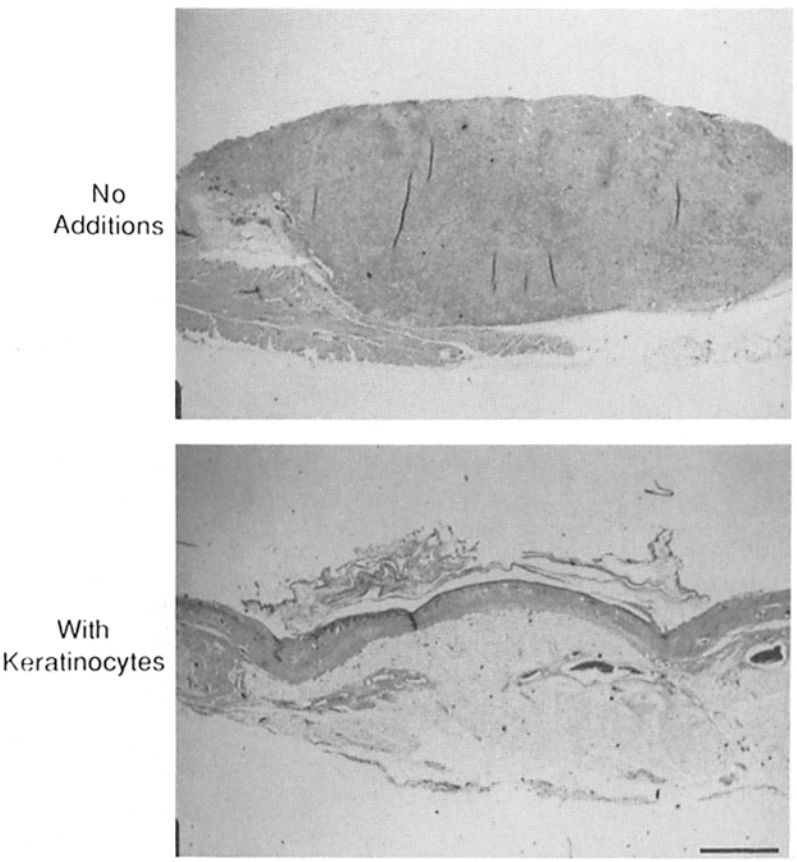

Figure 8. Histology of partially reconstituted syngeneic skin consisting of *neu* transformants grafted with or without keratinocytes. Mice were killed 4 w after grafting. Bar, 120 μm.

ous transformants, tumorigenicity was assayed also by subcutaneous injection of cells into nude mice. Even in this environment, bFGF-transformed melanocytes did not grow as tumors (Table IV). In two cases, small islands of pigmented cells were found subcutaneously at the presumed site of injection (data not shown). In contrast, rapidly expanding tumors were formed by melanocytes that had been transformed by the *myc* and *Ela* oncogenes (Table IV). No tumors could be detected after subcutaneous injection of these cells into syngeneic mice (data not shown), consistent with the lack of tumorigenicity after grafting (Table III).

Table IV. Tumor Growth after Subcutaneous Injection of Transformed Cells into Nude Mice

Cells	Tumors
L-B10.BR-*bFGF*	0/7
L-B10.BR-*p53*	0/5
L-B10.BR-*myc*	3/3 (5 g)
L-B10.BR-*E1a*	2/3 (0.25 g)
L-B10.BR-*ras*	3/3 (>1 g)
L-B10.BR-*neu*	7/7 (>1 g)
L-B10.BR-*neu* (4×10^5)	2/2 (6 g)
" + K	3/3 (3 g)
" + F	2/2 (1.5 g)
L-B10.BR-*neu* (5×10^4)	2/3 (1 g)
" + K	2/3 (0.25 g)
" + F	2/3 (0.8 g)
L-B10.BR-*neu* (5×10^3)	0/3
" + K	3/3 (0.7 g)
" + F	1/3 (0.6 g)

L-B10.BR melanocytes (5×10^5, except where specified) transformed with the indicated genes were injected subcutaneously into nude mice. Animals were periodically examined for macroscopic tumors and were killed 60 d after injection. The tumors were fixed in formalin for histological examination. The number of tumors per total number of injections is given as well as the average fresh tumor weight. *neu*-transformed melanocytes in the indicated numbers were also admixed with fixed numbers of B10.BR primary keratinocytes (*K*; 2×10^6) or dermal fibroblasts (*F*; 8×10^6) before injection.

Melanocytes that had been transformed with the *ras* and *neu* oncogenes produced rapidly expanding tumors (Table IV). These grew faster in nude mice than in similarly injected syngeneic animals (data not shown). Most of these tumors contained some pigmented regions. Melanocytes transformed with the *neu* oncogene were also tested for tumorigenicity after having been mixed with keratinocytes and/or dermal fibroblasts (Table IV). Decreasing numbers of *neu* transformants were injected together with a constant number of the other cells. Even under conditions where tumors were substantially delayed (by injection of as few as 5×10^3 transformants per mouse), no inhibitory effects of added keratinocytes were detected; rather, keratinocytes enhanced the formation of subcutaneous tumors (Table IV).

Discussion

Melanomas are highly variable with respect to chromosomal aberrations and expression of activated oncogenes, growth factors, growth factor receptors, and cell surface antigens (Houghton et al., 1982; Pathak et al., 1983; Dracopoli et al.,

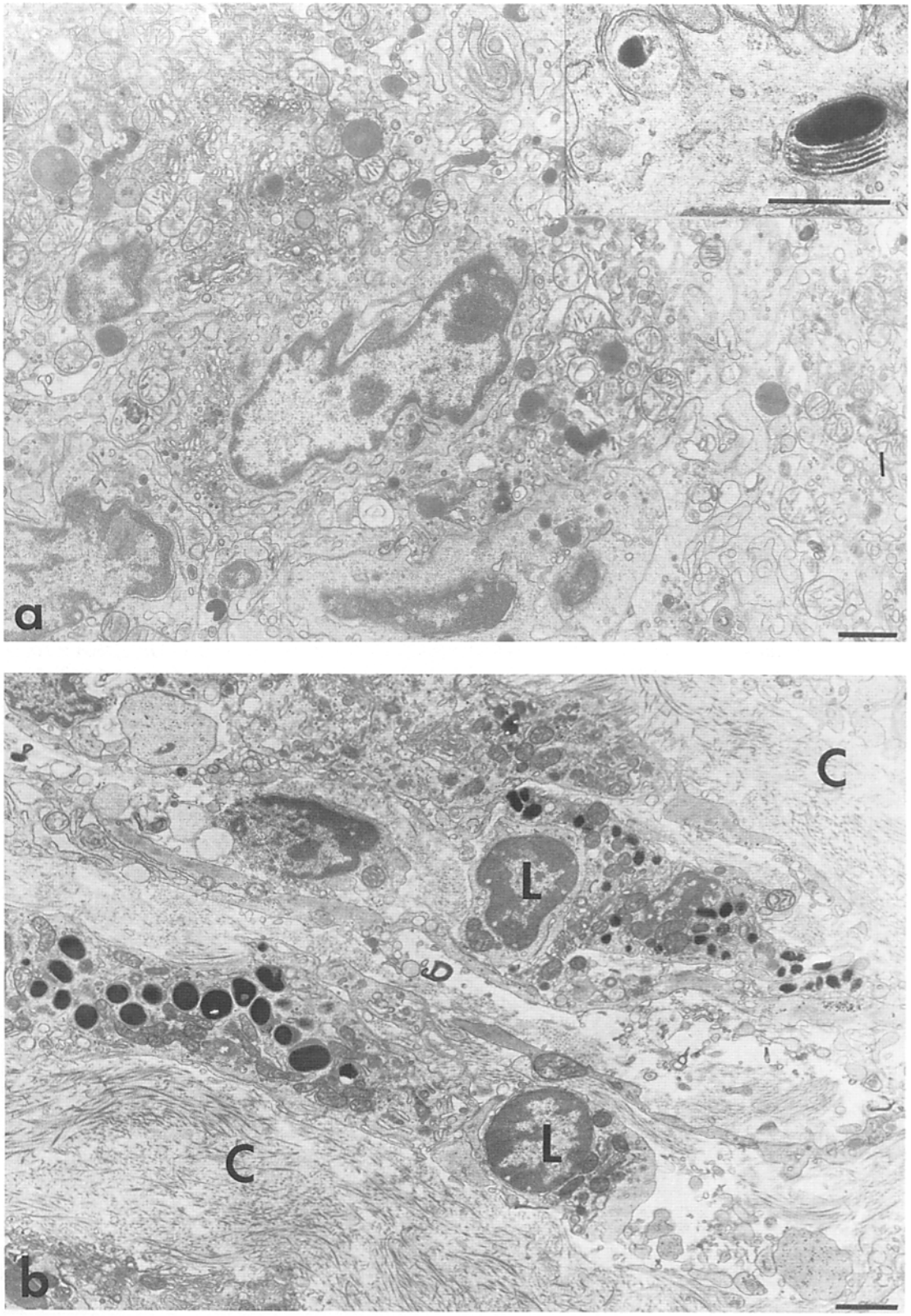

Figure 9. Neu-transformed L-B10.BR melanocytes in the dermis of incompletely and completely reconstituted syngeneic skin. In the absence of the epidermal component, the dermal connective tissue was displaced by tumor cells (*a*), whereas, in the presence of keratinocytes, the dermis was normally stratified and contained some melanotic pigment cells (*b*). Infiltrating lymphocytes (*L*) are seen in close apposition to melanocytes. (*Insert*) Evidence of residual melanogenesis in the grossly amelanotic transformed cells in vitro before grafting. *C*, Collagen bundles. Bars, 1 μm.

1985, 1989; Mukai and Dryja, 1986; Real et al., 1986; Westermark et al., 1986; Ellis et al., 1987; Holzmann et al., 1987; Shin et al., 1987; Yamaguchi et al., 1987; Cowan et al., 1988; Adelaide et al., 1988; Linnenbach et al., 1988; Raybaud et al., 1988; Richmond et al., 1988). In contrast, a characteristic that appears to be common at least to human metastatic melanomas is autonomous growth in vitro, independent of exogenous bFGF and cAMP. This trait appears to be acquired in part through the abnormal expression of bFGF (Halaban et al., 1988*b*), the natural growth factor for human melanocytes normally supplied by keratinocytes and possibly fibroblasts (Halaban et al., 1988*a*). Because bFGF is not expressed in normal melanocytes (Halaban et al., 1988*b*) and members of the FGF family are so far the only known natural mitogens for human melanocytes among at least 40 other factors tested (Halaban, 1988), the aberrant expression of bFGF by metastatic melanomas must represent a critical event in melanocyte transformation. bFGF may have two functions in promoting the development of melanomas: (*a*) it may play a role in the uncontrolled proliferation of melanocytes by releasing the cells from dependence on neighboring keratinocytes and fibroblasts; and (*b*) because of its angiogenic properties, it may induce capillary growth required for the sustenance of solid tumors (Folkman, 1985).

In the experiments described here, independence of melanocyte growth from exogenous bFGF was acquired within days of infection with a bFGF recombinant retrovirus, with concomitant production of biologically active bFGF at levels similar to those observed in human melanomas (Halaban et al., 1988*b*). Remarkably, constitutive expression of bFGF was associated with complete loss of differentiated properties and acquisition of a fibroblastoid phenotype, which is also seen in human melanoma cells grown in vitro. Since similar effects are not induced by a continuous supply of exogenous bFGF (our unpublished observations) and all other known mitogens, such as TPA or isobutylmethyl xanthine, stimulate rather than extinguish melanogenesis (Halaban et al., 1983), it is likely that loss of differentiated phenotype is due to activation of intracellular bFGF receptors by endogenous bFGF. This implies that mitogenic stimulation of melanocytes by exogenous growth factor is coupled to a signaling pathway activating genes controlling pigmentation and that continuous stimulation of that pathway is required to maintain melanocyte differentiated functions. Fibroblast transformation by the *sis* oncogene appears to be mediated through aberrant, intracellular stimulation of the PDGF receptor rather than through receptor stimulation from the outside (Keating and Williams, 1988). An analogous mechanism is more difficult to envision with bFGF, a ligand that lacks the signal peptide (Abraham et al., 1986) which would put it in the same subcellular compartment with its receptor. Nevertheless, indirect evidence for the activation of intracellular pools of bFGF receptor by endogenous bFGF was provided in the human melanoma system in which neutralizing antibodies to bFGF as well as anti-phosphotyrosine antibodies—the bFGF receptor is a tyrosine protein kinase (Lee et al., 1989)—inhibited growth when injected into the cells but not when supplied in the culture medium (Halaban et al., 1988*b*).

Aberrant expression of bFGF in vitro, however, is not sufficient to render melanocytes tumorigenic in vivo. In fact, the transformed cells reverted to the untransformed phenotype when placed into the cutaneous environment of host animals. To what extent they continued to express bFGF in this environment is not known. Likewise, the mediators of the phenotypic reversion of bFGF transformants are not known. Since comparable effects were seen in syngeneic and immunodeficient mice, the immune system is unlikely to be involved. Also, keratinocytes or dermal fibroblasts appeared not to be required for reversion of the bFGF transformants when these cells were grafted onto granulation tissue. It is possible that in vivo melanocyte turnover is slowed down and the bFGF produced by the transformants is released into the extracellular matrix and thus stimulates the plasma membrane receptors which then trigger the expression of differentiated functions. Suppression of tumor growth by a developing cutaneous environment has been reported for B16 murine melanoma cells injected into murine embryos (Gerschenson et al., 1986). In that system, a diffusable melanoma inhibitor was shown to be expressed transiently from gestational ages 10–14 d. The microenvironment in adult organs may also influence pigmentation and tumor growth as observed experimentally with B16 melanoma in mice (Price et al., 1988; Nicolson and Dulski, 1985) and clinically with human metastatic melanomas.

Our results are consistent with those of others who have shown that transfection of fibroblasts with a cDNA for bFGF can lead to morphological transformation in vitro (Sasada et al., 1988; Neufeld et al., 1988). Only high expression of transfected bFGF leads to tumorigenicity by fibroblasts (Quarto et al., 1989). However, when bFGF cDNA is transfected after the addition of a signal sequence, producing a chimeric protein containing a secretory signal peptide at its amino terminus, tumorigenic conversion of fibroblasts is induced even at low to intermediate levels of expression (Rogelj et al., 1988; Blam et al., 1988). Tumorigenic conversion of fibroblasts at low levels of growth factor expression has also been obtained with hst/K-FGF, a transforming oncogene from the FGF family which produced a secretory form of bFGF, suggesting that secreted FGF has a higher tumorigenic potential than the cell-associated form, possibly acting through an autocrine control loop (Quarto et al., 1989).

The results obtained by others with secreted forms of FGF are relevant to the newly discovered bFGF-related oncogenes, all of which contain a signal sequence and are released as diffusible factors (Delli-Bovi et al., 1987, 1988; Yoshida et al., 1987; Zhan et al., 1988; Marics et al., 1989). Two members of this family, hst/K-FGF and int-2, both with chromosomal assignment 11q13, were found to be coamplified in a conjunctival melanoma (one out of eight primary and metastatic melanomas tested; Adelaide et al., 1988). Because, like bFGF, hst/K-FGF is also a potent mitogen for normal cutaneous melanocytes (Halaban et al., 1988*b*), expression of these FGF-like growth factors probably gives growth advantage to a conjunctival melanoma as well.

The bFGF- and TPA-dependent, normally differentiated murine melanocytes were easily transformed to an autonomous mode of growth not only by bFGF cDNA but also by other oncogenes. Since endogenous bFGF was not induced in any of these oncogene transformants, it appears that transformation to growth factor independence of these melanocytes can be accomplished by several pathways that may converge at a critical but so far unidentified step. The variously transformed melanocytes exhibited differences in morphol-

ogy in vitro as well as substantial differences in behavior in vivo in response to a variety of environments. In general, oncogene-transformed melanocytes grew rapidly as tumors when injected subcutaneously into nude mice. However, when tested by subcutaneous injection or by grafting onto syngeneic, immunocompetent mice, only *ras*-transformed melanocytes were able to form tumors irrespective of their environment.

Neu-transformed melanocytes presented an interesting example of tumor growth modulated by surrounding normal cells. Addition of keratinocytes at the time of grafting was sufficient to inhibit tumor formation in syngeneic animals. In contrast, dermal fibroblasts promoted rather than inhibited growth. Since inhibition by keratinocytes was not observed in nude mice, it is likely that tumor growth in immunocompetent syngeneic mice was prevented as the result of an interplay between the keratinocytes and the immune system. It is known, for instance, that keratinocytes, which are highly phagocytic cells, display class II histocompatibility antigens in vitro after treatment with interferon (Wikner et al., 1986), suggesting that keratinocytes could function as antigen-presenting cells. If so, keratinocytes in immunocompetent mice may facilitate immune recognition of *neu* transformants. This interpretation would be consistent with the close apposition of lymphocytes to residual melanocytes, observed by electron microscopy and by the clinical observation of the slow initial growth of melanomas while still confined to the epidermis.

Carcinoma formation by *ras*-transformed primary keratinocytes can be suppressed by concomitant grafting of normal dermal fibroblasts (Dotto et al., 1988). Those results and the results reported here raise the general possibility that heterotypic cell interactions play an important role in regulating tumor development. The underlying mechanisms are likely to be different in the two cases. Since carcinoma inhibition by dermal fibroblasts can be observed also in nude mice, the immune system in this case is unlikely to be involved (Dotto, G. P., unpublished observation).

Cells transformed by the *Ela* or *myc* oncogenes were tumorigenic in nude but not in syngeneic mice irrespective of the presence of normal cutaneous cells, suggesting that the immune system can recognize these transformants and block their growth probably because of the presence of new surface antigens or new combinations of surface antigens. A precedent is the induction of class II histocompatibility antigens in human melanocytes after transformation by the *ras* oncogene (Albino et al., 1986). In addition, a number of melanoma-associated antigens have been described (Herlyn et al., 1987; Houghton et al., 1982; Holzman et al., 1987; Hotta et al., 1988; Yamaguchi et al., 1987).

The most effective transforming agent in our system was the v-*ras* oncogene. Work by others has indicated that c-*ras* activation may have occurred in 10–15% of human metastatic melanomas (Albino et al., 1984; Sekiya et al., 1984; Padua et al., 1985; Gerhard et al., 1986; Albino, 1988; Raybaud et al., 1988). Direct transformation of normal human melanocytes by v-*ras* oncogenes resulted in a partially transformed phenotype, including induction of new surface antigens and ability to grow in soft agar (Albino et al., 1986; Albino, 1988). However, other markers of transformation, such as growth factor independence and loss of pigment forming ability, were not observed, suggesting that transformation of human melanocytes by *ras* oncogenes alone was not sufficient to induce a fully transformed phenotype (Albino et al., 1986). Recently, results similar to ours were reported by Wilson et al. (1989), who showed that transformation of a nontumorigenic line of murine melanocytes with the v-Ha-*ras* oncogene resulted in tumorigenic conversion (as detected in nude mice) together with growth factor independence and amelanosis in vitro. Extinction of differentiated functions was observed also after transformation of chicken retinal melanocytes with Rous sarcoma virus (Boettiger et al., 1977), and uveal melanomas were induced by direct injection of feline sarcoma virus into the anterior chamber of the eyes of newborn kittens (Albert et al., 1981).

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