PHENOTYPIC HETEROGENEITY OF MELANOMA

Relation to the Differentiation Program of Melanoma Cells

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Tumor cell heterogeneity is a fundamental characteristic of cancer that has profound implications for cancer therapy (1–3). Melanoma, a tumor arising from pigmented cells, is particularly accessible for the study of tumor heterogeneity. Metastatic melanoma cells can vary widely in their degree of pigmentation, cell morphology, and growth rate (4, 5), and these differences can be observed in different metastases and even among the various cells within individual lesions of patients with melanoma. There are a number of biological factors that could regulate the phenotypic heterogeneity observed in melanoma cells. One possibility is that melanoma heterogeneity reflects a corresponding diversity in the phenotype of normal cells undergoing melanocyte differentiation. According to this view, patterns of gene expression change as cells progress through distinct stages in the melanocyte lineage. To pursue this idea, we analyzed the antigenic phenotype of melanocytes and melanoma (6). Antigens could be grouped into four categories: (*a*) not present on melanocytes, (*b*) expressed by fetal and newborn melanocytes but not adult, (*c*) expressed by adult but not fetal/newborn melanocytes, and (*d*) equally expressed on fetal, newborn, and adult melanocytes. Antigens not expressed on melanocytes but present on undifferentiated melanoma cells were presumed to be markers present on early precursor cells in the melanocyte pathway. Antigens expressed on fetal and newborn but not adult melanocytes marked an intermediate phase of melanocyte differentiation. Antigens expressed on adult but not fetal or newborn melanocytes were considered to be late markers. By comparing antigen expression with other differentiation characteristics including pigmentation, cell morphology, and tyrosinase activity, the phenotype of melanoma cell lines was found to correspond to early, intermediate, or late stages of melanocyte differentiation (6). In the present study, we have investigated the regulation of differentiation markers by melanoma cells. Clones were established from two melanoma cell lines in order to isolate individual and distinct phenotypes that represented phenotypes present in the original tumor. These clones provide stable, relatively homogeneous cell popu-

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lations that have allowed the analysis of the coordinate expression of phenotypic characteristics by melanoma cells.

Materials and Methods

Cell Culture Studies. Melanoma cell lines were established as previously described (7). Parental melanoma cultures and cloned cell lines were maintained in T-25 or T-75 flasks (Falcon Labware, Oxnard, CA) in Eagle's minimum essential medium supplemented with 7.5% FCS, 2 mM glutamine, 1% nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium). Cultures were maintained at 37°C in 5% CO_2. Medium from cultures was changed every 3–4 d and cultures were passaged every 2–3 wk with trypsin (1 mg/ml) and EDTA (0.2 mg/ml) in HBSS. Cultures were tested at regular intervals for mycoplasma and any contaminated cultures were discarded.

Parental cultures of SK-MEL-131 and SK-MEL-23 were initially cloned by limiting dilution in 96-well plates (Falcon Labware). All primary clones were subcloned at least one to two more times before use in experiments. Subclones of SK-MEL-23 were diluted in complete medium to give a concentration of 3 cells/ml, and then 0.1 ml was added to each well (at 0.3 cell/0.1 ml) in a series of 96-well plates. Individual wells were evaluated under a microscope ~18 h after plating of cells and wells containing only a single cell were scored and observed for outgrowth of subclones. The nonpigmented clone 22.a was derived from a BALB/c *nu/nu* tumor established from the pigmented clone 22 of SK-MEL-23, and was subcloned as described above. Clone 22.a expressed human HLA but not mouse H-2 antigens or the mouse leukemia virus antigen glycoprotein (gp)[1] 70. Subclones of SK-MEL-131 were established as previously described (8).

For induction studies, cells were cultured in T-25 flasks in complete medium supplemented with 12-*O*-tetradecanoyl-phorbol-13-myristate acetate (PMA) 1.6×10^{-8} M (PL Biochem, Division of Pharmacia, Inc., Piscataway, NJ), cholera toxin 10^{-9} M (Sigma Chemical Co., St. Louis, MO) or a combination of PMA and cholera toxin.

For determination of doubling times, 2.5×10^5 cells were plated into each well of Costar 3424 plates (Costar, Cambridge, MA) and trypsinized cells were counted from duplicate wells with a hemocytometer daily for 6–8 d. Time of doubling was determined from a least squares regression fit of cell number versus time during logarithmic growth phase.

Serological Studies. Table I lists the characteristics and source of antibodies used for serologic studies (6, 9–14).

The anti–mouse Ig and protein A hemadsorption assays were performed as previously described (15–17). Antibodies, diluted serially, were incubated with cells cultured in Falcon 3040 Microtest II plates (Falcon Labware) at room temperature for 1 h. Cells were then washed three times with PBS and 5% (vol/vol) γ-globulin–free FCS in PBS and incubated for 45 min with a 0.2% (vol/vol) suspension of human erythrocytes conjugated to anti–mouse Ig or protein A, washed again, and scored for rosetting. Methods for indirect immunofluorescence assays have been reported previously (18). Cells cultured in Falcon 3040 Microtest II plates were fixed with a methanol/acetone mixture (1:1) at 4°C for 10 min. Plates were incubated with antibodies and then washed as described above. Cells were incubated with goat anti–mouse Ig conjugated to FITC (Cappel Laboratories, Cochranville, PA) for 45 min, washed, and examined with epifluorescence.

Tissue Studies. For evaluation of original tumor tissues, 6–8-μm sections were stained with hematoxylin and eosin, or Grimelius (silver nitrate) stain for melanin (19).

Pigmentation and Tyrosinase Activity. Pigmentation was estimated by intensity of brown or black pigment in cell pellets and confirmed by determining absorbance at 470 nm of supernatants from 2×10^6 cells solubilized overnight in 0.1 N NaOH/10% dimethylsulfoxide. Absorbance by cell pigment was compared to absorbance by a solubilized melanin

[1] *Abbreviations used in this paper:* CALLA, common acute lymphoblastic leukemia antigen; EGFR, epidermal growth factor receptor; gp, glycoprotein; mCSP, melanoma chondroitin sulfate proteoglycan.

TABLE I
Characteristics and Source of Antibodies in Serologic Studies

mAb	Antigen detected	Reference
AO10	gp110	6, 9
225	gp 170; epidermal growth factor receptor (EGFR)	10, 23
L243	gp 33, 27; Ia antigens (HLA-DR)	11, 27
B5.2	gp 240, >400; melanoma chondroitin sulfate proteoglycan (mCSP)	6, 12
M111	gp 110	6, 12
NL-1	gp 100; common acute lymphoblastic leukemia antigen (CALLA)	6, 13
C350	gp 180	
CF21	Antigen of mature melanosomes	
TA99	gp 75; antigen of mature melanosomes	14
Mel 1	Heat labile	6, 15
M144	Heat labile	6, 12

standard (Sigma Chemical Co.). Tyrosinase activity was determined by the method of Pomerantz (20), modified as previously described (21). Measurements of tyrosinase were performed in triplicate from cells extracted in PBS, 0.5% Nonidet-P40 (NP-40), 50 μM tyrosine, 50 μM DOPA and 5 μCi/ml 3',5'-[^{3}H]tyrosine (New England Nuclear, Boston, MA). Tyrosinase activity was expressed as the ratio of ^3H_2O formed by (10^6 test cells)/(10^6 tyrosinase-negative renal cancer cell line SK-RC-6). Ratios of ^3H_2O calculated per milligram of protein of cell extracts gave results within 20% of ratios calculated from cell number.

Radioimmunoprecipitation. Analysis of immunoprecipitates was performed as previously described (22). Cultured cells were labeled with [^{3}H]glucosamine (New England Nuclear) for 72 h. 0.5% NP-40 cell lysates were prepared and equivalent ^{3}H counts per minute (determined by precipitation at 4°C with 10% vol/vol TCA) of lysate were used for immunoprecipitation with each antibody. Immunoprecipitation was carried out with monoclonal antibody plus rabbit anti-mouse Ig and PA-Sepharose (Pharmacia Inc.) and was analyzed on 9% SDS-PAGE.

Results

Serological Typing of Melanomas: Characterization of Antigenic Phenotypes and Selection of Cell Lines for Subcloning

28 melanoma cell lines from 26 patients were evaluated for antigen expression, tyrosinase activity, cell morphology, and pigmentation (Fig. 1). 11 antigen systems were analyzed in the present study because they define subsets of melanomas, have distinct patterns of expression on fetal, newborn, and adult melanocytes, and have been interpreted to map to different stages of melanocyte differentiation (early, intermediate, or late). Antigens AO10, epidermal growth factor receptor (EGFR), melanoma chondroitin sulfate proteoglycan (mCSP), and Ia are early markers of melanocyte differentiation, antigens M111 and common acute lymphoblastic leukemia (antigen) (CALLA) are markers of an intermediate stage, and antigens C350, CF21, TA99, Mel 1, and M144 are late markers (Table I) (6, 23).

Two melanoma cell lines (highlighted in Fig. 1) were selected for more detailed studies: (*a*) SK-MEL-131, derived from patient FD, has an epithelioid morphol-

MELANOMA	ANTIGEN SYSTEM											MORPH.	PIGMENT	TYR'ASE
	A010	EGFr	Ia	mCSP	M111	CALLA	C350	CF21	TA99	Mel 1	M144			
SK-MEL-31	++	+	−	++	−	−	−	−	−	−	−	E	−	1.0
SK-MEL-186	+++	++++	−	++++	−	−	−	−	−	−	−	E	−	1.0
SK-MEL-187	−	++++	++++	+++	−	−	−	−	−	−	−	E	−	1.0
SK-MEL-178	+	++++	++++	++++	−	+	−	−	−	−	−	E	−	1.0
SK-MEL-37	+	+	++++	++++	+	−	−	−	−	−	−	E	−	1.0
DX-3	+	−	++++	+++	−	−	−	−	−	−	−	E	−	1.0
SK-MEL-131	++	++++	++++	+++	−	−	−	−	−	−	−	E	−	1.0
SK-MEL-170	−	++++	++++	++	−	−	−	−	−	−	−	E-S	−	1.0
SK-MEL-173	+	++++	−	+++	++++	−	−	−	−	−	−	E-S	−	1.9
SK-MEL-176	−	−	++++	++++	++++	−	−	−	−	−	−	S	−	1.4
SK-MEL-81	−	+	−	++++	−	−	−	−	−	−	−	E-S	−	1.1
DX-5	−	−	++++	+++	++	−	−	−	−	−	−	E-S	−	1.2
SK-MEL-118	++		+++	++	++	++	−	−	−	−	−	E-S	−	1.0
SK-MEL-13	−	−	+++	++	++++	++++	+++	−	−	−	−	S	+or−	1.9
SK-MEL-30	−	−	−	+	+++	++++	++++	+++	++++	−	−	S	++	5.5
SK-MEL-29	−	−	+	−	−	−	+	−	−	+++	+	S	+or−	1.6
DX-2	−	−	−	−	−	++++	++++	+++	++++	++	−	S	+or−	1.8
SK-MEL-28	−	−	+	+++	+++	+++	++	−	−	++	−	S	+or−	1.4
SK-MEL-75	−	−	++++	+++	−	++++	++++	+++	+	+	−	S	+or−	1.6
MeWo	−	−	−	+	++++	++++	+	++	++	+	+	S	+or−	1.7
SK-MEL-127	−	−	−	+++	−	++	++	+++	+++	++	++	S-D	++	4.1
SK-MEL-130	−	−	+	−	++	−	++++	+++	+++	+	+	S	+	2.2
SK-MEL-64	−	−	−	+	++++	+++	++++	+++	++++	+	−	S	++	3.0
SK-MEL-23	−	−	−	+	++++	−	++++	++++	++++	+++	−	S-D	+++	13.3
SK-MEL-19	−	−	−	+	+++	−	++++	+++	++++	+++	+	S-D	++	7.8
SK-MEL-110	−	−	−	−	++	−	+++	+++	+++	+++	−	S-D	+or−	2.5
SK-MEL-21	−	−	−	−	+	−	++++	++	+++	+++	+	S	++	8.4
SK-MEL-188	−	−	−	−	−	−	++++	++++	++++	++	+	S	+++	7.6

FIGURE 1. Serologic typing of melanoma cell lines for melanocyte differentiation markers. Antigen expression is graded by titers: +, 1/200 to 1/1,000; ++, >1/1,000 to 1/10,000; +++, >1/10,000 to 1/100,000; ++++, >1/100,000. Morphology (*MORPH.*): *E*, epithelioid; *S*, spindle; *D*, dendritic. Pigmentation is determined as outlined in Materials and Methods. Tyrosinase activity is expressed as the ratio of melanoma/control renal cancer culture (SK-RC-6).

ogy, lacks pigmentation, and expresses early melanocyte markers, and (*b*) SK-MEL-23 from patient AP contains a mixture of spindle and polydendritic cells, is deeply pigmented, expresses high tyrosinase activity and late markers, and is representative of cells at a late stage of melanocyte differentiation.

Heterogeneity in Tumors from Patients FD and AP

There is a high degree of variability in cell morphology, pigmentation, and expression of melanocyte differentiation antigens in tissue sections of metastatic melanoma lesions (4, 12, 14, 24). Characteristically, individual differentiation antigens are detected on only a proportion of cells within tumor specimens. For example, we have found that Ia, mCSP, M111, CF21, TA99, and C350 antigens are expressed on distinct subsets of melanoma cells in reactive tumors (12, 14). To investigate this phenotypic heterogeneity of melanoma, we examined the phenotype of cells in the original tumor lesions of patients FD and AP. The cell line SK-MEL-131 was established from an axillary lesion resected from patient FD and the cell line SK-MEL-23 was derived from a surgically resected metastatic tumor in the axilla of patient AP. Tissue sections from paraffin-embedded blocks of these lesions were examined for cell morphology, pigmentation, and antigen expression. Substantial variability in cell shape and pigmentation was observed. Each lesion contained a mixture of epithelioid and spindle cells (Fig. 2*A*) and

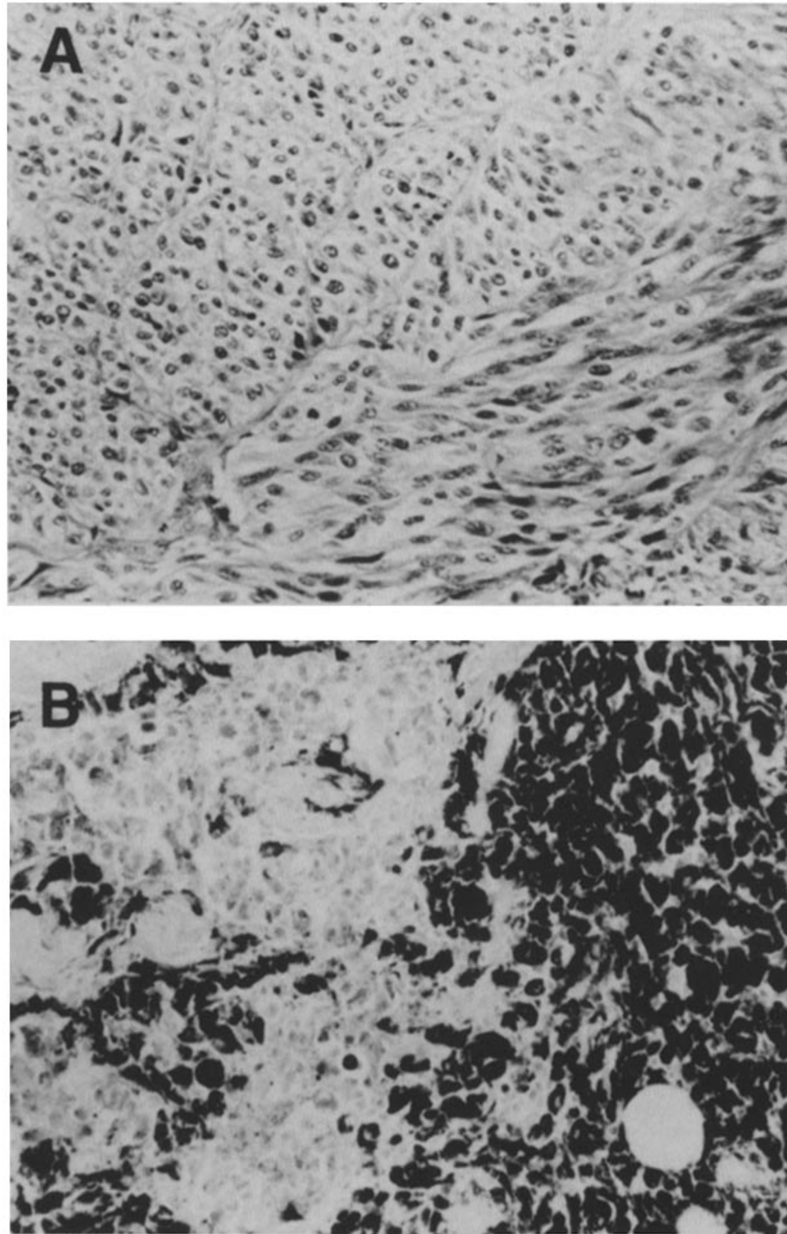

FIGURE 2. Histology of representative areas of melanoma from patients FD and AP (*A*) Tissue section from an axillary lesion of patient FD demonstrating a mixture of epithelioid (*top-left*) and spindle (*bottom-right*) tumor cells (stained with hematoxylin and eosin, × 200). (*B*) Tissue section from an axillary lesion of patient AP demonstrating a mixture of pigmented and nonpigmented tumor cells (Grimelius strain, × 200). SK-MEL-131 was derived from the lesion in *A* and SK-MEL-23 from the lesion in *B*.

areas of pigmented and nonpigmented tumor (Fig. 2*B*). We were unable to measure antigen expression, inasmuch as none of the determinants of differentiation antigens that we tested were stable after paraffinization-deparaffinization.

Phenotypes of Clones from Melanoma Cell Lines SK-MEL-131 and SK-MEL-23: Antigen Expression, Pigmentation, and Tyrosinase Activity

We investigated whether the various phenotypes observed in tumors of patients FD and AP were expressed in cloned cell lines derived from these lesions. Clones were established from early passage cells frozen shortly after the original tumor cells were established in culture. The cell line SK-MEL-131 from patient FD was cloned at passage 7 and SK-MEL-23 from patient AP was cloned at passage 8. Each clone was subcloned two to three times and then evaluated for expression of antigens and other differentiation-related characteristics.

17 clones were studied from SK-MEL-131 and 19 clones from SK-MEL-23. Clones from each parental cell line varied in cell morphology and pigmentation (Figs. 3 and 4). Differentiation traits were expressed in a coordinate fashion on cloned cell lines. Expression of early antigens correlated with an epithelioid or spindle shape, an absence of pigment, and low or no detectable tyrosinase activity. Expression of late markers corresponded to a spindle or dendritic morphology and presence of pigment and tyrosinase activity. Fig. 5 lists the characteristics of representative clones derived from SK-MEL-131 and SK-MEL-23. Clones from SK-MEL-131 ranged from early to intermediate stage of melanocyte differentiation, and clones from SK-MEL-23 ranged from intermediate to late stage. Epithelioid clones from SK-MEL-131 (e.g., clone 1.36-1-5) expressed only early melanocyte markers and no detectable tyrosinase activity, while spindle shaped clones (e.g., clone 3.44) expressed intermediate markers, tyrosinase activity, and light pigmentation. 18 of 19 clones of SK-MEL-23 were pigmented, had moderate to high levels of tyrosinase activity, and expressed late melanocyte markers. The 19th subclone of SK-MEL-23, clone 22.a, was nonpigmented and had no detectable tyrosinase activity. Clone 22.a was derived after passage of the pigmented parental clone 22 through *nu/nu* mouse (see Materials and Methods). Clone 22 expressed the late marker C350 and the melanosomal antigens TA99, and CF21, but not the early markers Ia, mCSP, and M111. In contrast, clone 22.a did not express late markers (C350$^-$, TA99$^-$, and CF21$^-$) but did express Ia, mCSP, and M111.

To evaluate whether growth rate might be related to expression of differentiation traits, doubling times during logarithmic growth phase were measured in selected clones. The early stage clone 1.36-1-5 had a doubling time of 54 h and intermediate stage clones (clones 3.44, 22.a, and 6113) doubled every 48–54 h. Doubling times of late stage clones were 52 h (clone 22), 79 h (clone 19), and >120 h (clone 711). These results show that some melanoma clones corresponding to late stages of differentiation grow at a very slow rate, but the rate of growth of clones at other stages was similar. All subclones of SK-MEL-131 and SK-MEL-23 have been maintained for more than 2 yr in culture and phenotypes have remained stable over this period unless tissue culture conditions were manipulated (e.g., deprivation of serum, very high cell densities).

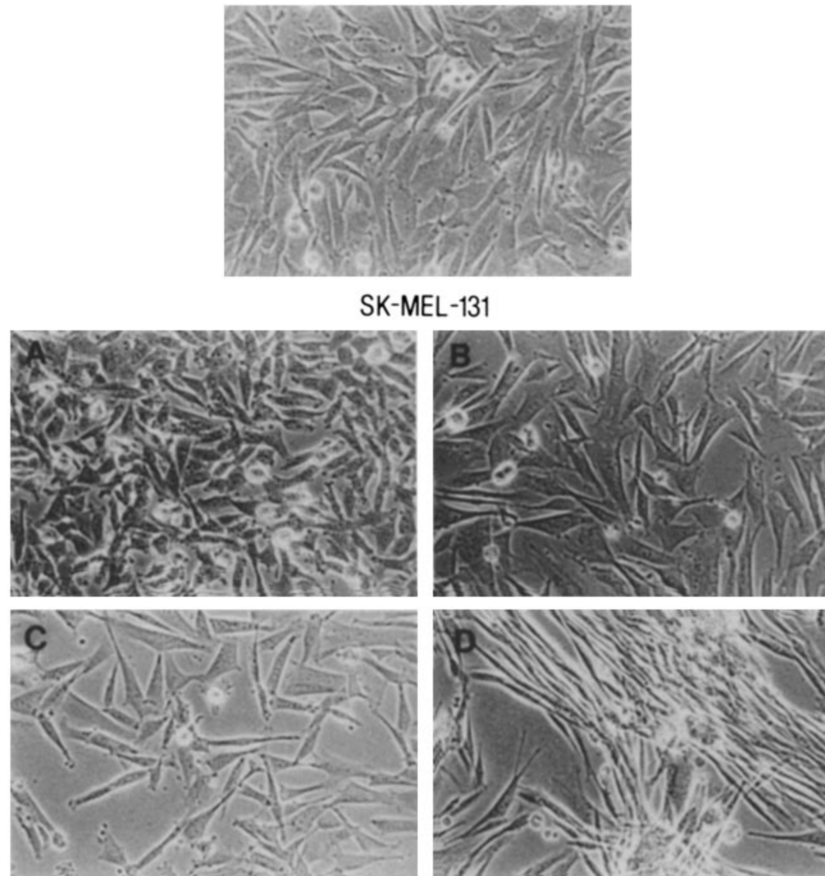

FIGURE 3. Parental cell line SK-MEL-131 and four subclones: (*A*) clone 1.36-1-5 contains epithelioid, nonpigmented cells; (*B*) clone 1-9 contains epithelioid, nonpigmented cells; (*C*) clone 2.45 contains epithelioid and spindle, nonpigmented cells; and (*D*) clone 3.44 contains spindle, lightly pigmented cells. A description of phenotypic traits of these clones is given in Fig. 5. × 360.

Further Analysis of the Differentiation Program of Melanoma Cells: Induction of Differentiation

To establish that differentiation characteristics on melanoma cells can be regulated in a coordinate fashion, four clones were evaluated in induction studies. Clones were selected that corresponded to early (clone 1.36-1-5), intermediate (clones 3.44 and clones 22.a), and late (clone 22) stages of differentiation. Each clone was cultured in the presence of the phorbol ester PMA, cholera toxin, or a combination of PMA and cholera toxin.

Each clone responded in a distinct manner to inducing agents. Clone 3.44 was induced to differentiate by PMA and clones 22.a and 22 by cholera toxin, whereas clone 1.36-1-5 was induced to undergo only minor changes in phenotype by either PMA or cholera toxin. Thus, each clone could be induced to undergo a shift in differentiation characteristics (Fig. 6), but the degree of alteration

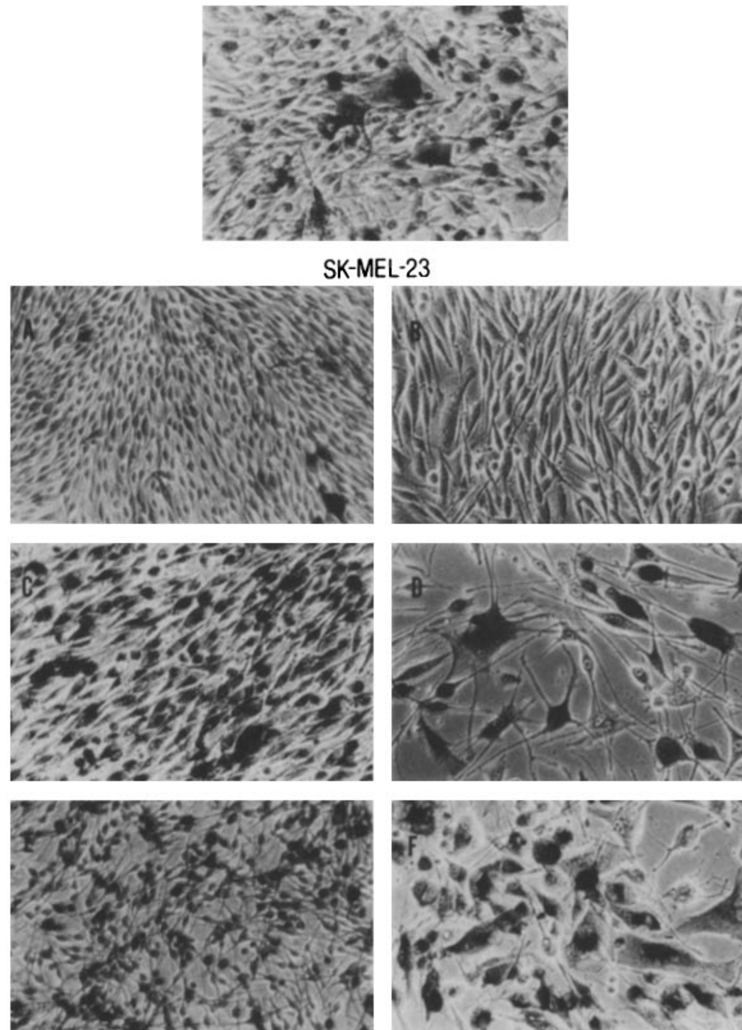

FIGURE 4. Parental cell line SK-MEL-23 and six subclones: (*A*) clone 6113, (*B*) clone 6115, (*C*) clone 22-1, (*D*) clone 15, (*E*) clone 19, (*F*) clone 711. Clones 6113 (*A*) and 6115 (*B*) contain spindle-shaped cells with light to moderate pigmentation. Clone 22-1 (*C*) contains deeply pigmented, spindle cells. Clones 15 (*D*), 19 (*E*), and 711 (*E*) contain dendritic or a mixture of spindle-dendritic cell with deep pigmentation. A description of phenotypic traits of *A*, *D*, and *F* is given in Fig. 5. × 360.

varied—only minimal changes were detected after induction of the early clone 1.36-1-5 by the combination of PMA and cholera toxin, but a major shift in the differentiation state of clone 22.a from intermediate to mature phenotype was observed during exposure to cholera toxin. Major alterations in phenotype were not clearly evident until after 2–3 wk. For example, during induction of clone 22.a, there was a gradual increase of pigment, appearance of C350 antigen between days 4 and 7, and appearance of antigens CF21 and TA99 between the

MELANOMA CLONES	ANTIGEN SYSTEM											MORPH.	PIGMENT	TYR'ASE
	AO10	EGFr	Ia	mCSP	M111	CALLA	C350	CF21	TA99	Mel 1	M144			
SK-MEL-131														
1-9	+++	++++	+++	+++	–	–	–	–	–	–	–	E	–	1.0
1.36-1-5	–	++++	+++	+	–	–	–	–	–	–	–	E	–	1.0
1-13	+++	++++	+++	+++	+++	–	–	–	–	–	–	E	–	1.2
2.45	–	–	+++	++	–	–	–	–	–	–	–	E-S	–	1.4
3.45	–	–	+++	+++	++++	+++	–	–	–	–	+	S	+	1.5
3.44	–	–	++++	+++	++++	++++	–	–	–	++	++	S	+	1.8
SK-MEL-23														
22.a	+++	–	++++	+++	++++	+++	–	–	–	+	–	S	–	0.9
6113	–	–	–	+++	+++	–	++++	+++	+++	++	++	S	++	9.9
8114	–	–	–	–	+++	–	++++	+++	+++	+++	++	S	+	6.9
22	–	–	–	–	–	–	++++	+++	++++	+++	–	S	++	12.4
7111	–	–	–	–	–	–	++++	+++	+	+++	+	D	++++	21.2
19	–	–	–	–	–	–	+++	+++	+++	+++	++	S-D	+++	34.1

FIGURE 5. Phenotypic characteristics of subclones of SK-MEL-131 and SK-MEL-23. See legend to Fig. 1 (antigen expression, morphology, pigmentation, and tyrosine activity).

second and third week (CF21 was expressed before TA99). Specifically, the following observations were made:

Parental cell lines SK-MEL-131 and SK-MEL-23. No changes in antigen expression were observed in SK-MEL-131 during incubation with PMA or cholera toxin, although PMA did induce an increase in the production of spindle-shaped cells. No changes were detected in the antigenic profile of SK-MEL-23 in the presence of either cholera toxin or PMA. Cholera toxin induced a polydendritic morphology and increased pigmentation in SK-MEL-23 cells, and this effect was inhibited by PMA.

Clone 1.36-1-5 (early stage). The combination of PMA and cholera toxin induced a decrease in the expression of early markers AO10, mCSP, and Ia, and a concurrent induction of the intermediate marker CALLA and the later marker C350 (Fig. 6). These changes in antigen expression were accompanied by the occasional appearance of dendritic cells, but the cells remained nonpigmented and tyrosinase-negative (Fig. 7*A*). Once clone 1.36-1-5 was induced with PMA and cholera toxin, the induced antigenic changes were maintained when PMA and cholera toxin were removed from the culture medium. No phenotypic changes were observed when clone 1.36-1-5 was exposed to either PMA or cholera toxin alone.

Clone 3.44 (intermediate stage). PMA induced long dendritic processes on the spindle-shaped clone 3.44 and augmented tyrosinase activity and melanin synthesis (Fig. 7*B*). These changes were accompanied by a change in antigen profile, with downregulation of the early markers mCSP and Ia and induction of the late markers C350 and CF21 (Fig. 6). After induction with PMA, the altered phenotype was maintained in the absence of PMA. Similar but less pronounced changes were observed in the presence of PMA and cholera toxin, and cholera toxin alone had no effect on the phenotype of clone 3.44.

Clone 22.a (intermediate stage). The most profound change in differentiation program was observed in clone 22.a during induction with cholera toxin. During induction, clone 22.a, which was nonpigmented and tyrosinase-negative, became pigmented and tyrosinase-positive (Figs. 7*C* and 8). Early and intermediate

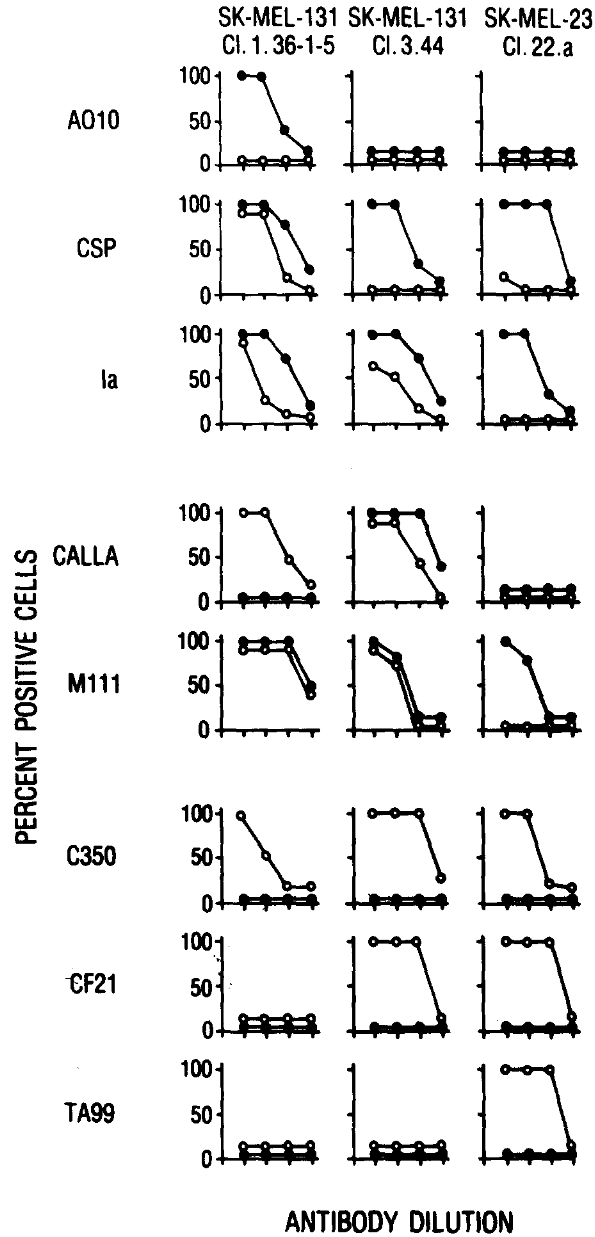

FIGURE 6. Serologic typing of subclones 1.36-1-5 (derived from SK-MEL-131 parental cell line), 3.44 (SK-MEL-131), and 22.a (SK-MEL-23) for expression of antigens AO10, mCSP (CSP), Ia, CALLA, M111, C350, CF21, and TA99 in induced (*solid circles*) and control (*open circles*) cultures. Clone (*Cl.*) 1.36-1-5 was induced with cholera toxin and PMA, clone 3.44 with PMA, and clone 22.a with cholera toxin as described in the text. Expression of antigens AO10, CSP, Ia, CALLA, M111, and C350 were tested by anti-mouse Ig assays: antibody dilutions were 1/1,000, 1/10,000, 1/100,000, and 1/1,000,000. Expression of CF21 and TA99 were tested by indirect immunofluorescence assays: antibody dilutions were 1/100, 1/1,000, 1/10,000, and 1/100,000.

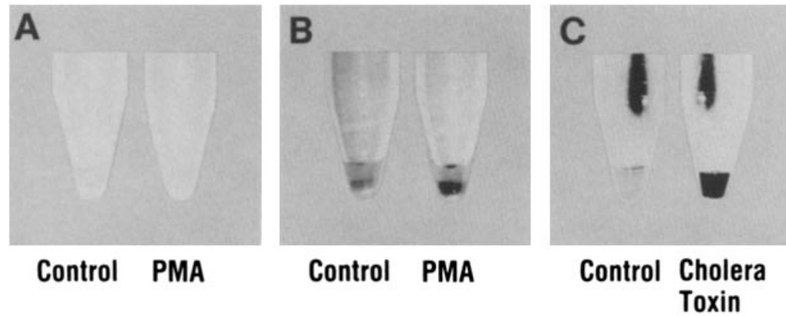

FIGURE 7. Cell pellets of subclones from control and induced cultures: (*A*) clone 1.36-1-5 shows nonpigmented cells and no change after induction with PMA; (*B*) clone 3.44 shows an increase in pigment when induced with PMA; and (*C*) clone 22.a becomes pigmented when induced with cholera toxin.

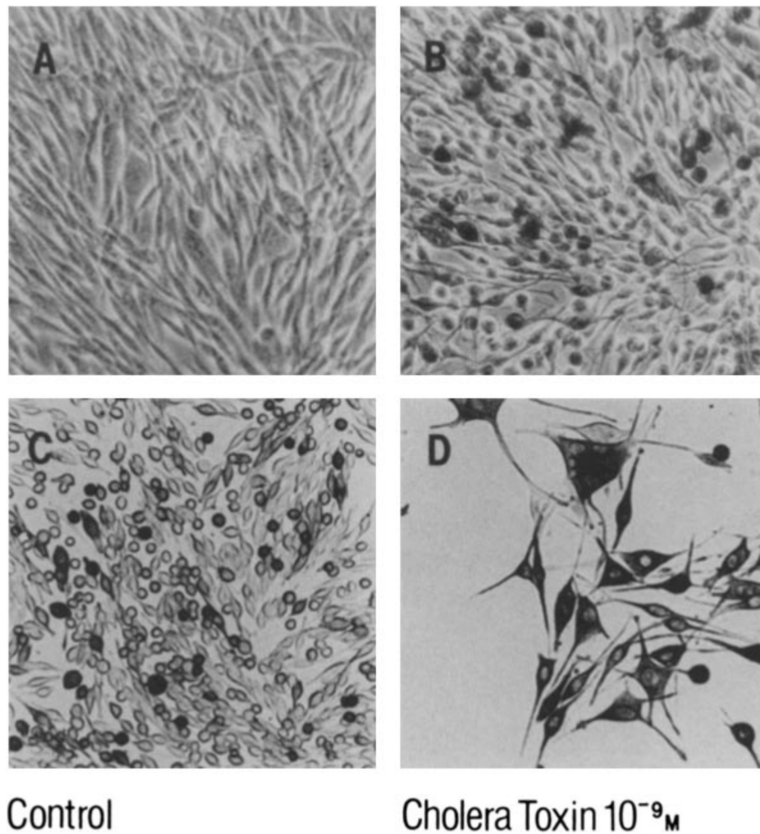

FIGURE 8. Induction of subclones 22.a and 22 of SK-MEL-23: (*A*) control culture of clone 22.a, (*B*) culture of clone 22.a induced with cholera toxin, (*C*) control culture of clone 22, (*D*) culture of clone 22 induced with cholera toxin. × 320.

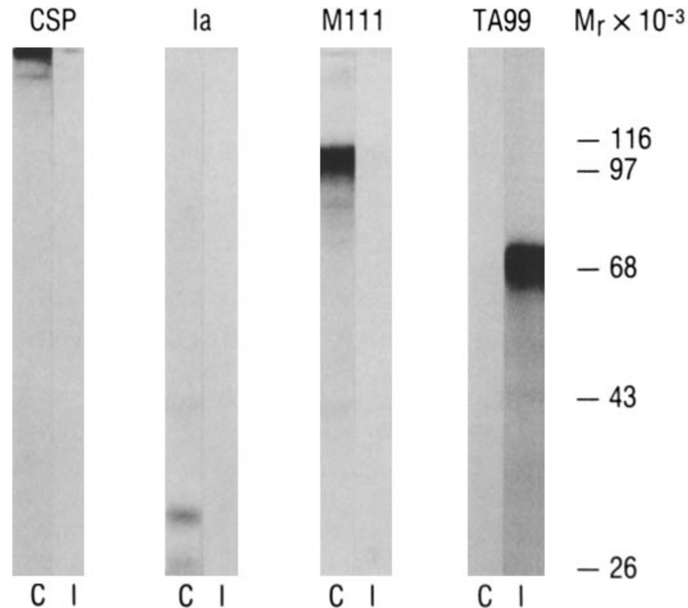

FIGURE 9. Immunoprecipitation of antigens from control (*C*) or induced (*I*) cultures of subclone 22.a of SK-MEL-23. Cultures were induced with cholera toxin (10^{-9} M). Cells were metabolically labeled with [^{3}H]glucosamine. CSP, gp240/>400; Ia, gp33/27; M111, gp110; TA99, gp75.

markers (mCSP, Ia, and M111) were downregulated while late markers (C350, CF21, and TA99) were induced (Fig. 6). These serologic results were confirmed by radioimmunoprecipitation of antigens from induced and control cells (Fig. 9). The change in differentiation program produced by cholera toxin was stable and was not reversed by removing cholera toxin from the culture medium. PMA alone and the combination of PMA and cholera toxin induced clone 22.a cells to become more dendritic, but there was no change in other traits.

Clone 22 (late stage). Cholera toxin induced clone 22 to become heavily pigmented and polydendritic (Fig. 8). A decrease in the expression of the melanosomal antigen TA99 was observed, but no other antigen changed expression (data not shown). The doubling time of clone 22 increased from 52 to >120 h after 21 d of exposure to cholera toxin. PMA alone and the combination of PMA and cholera toxin produced elongated, spindle-shaped cells but no other alteration, again suggesting that exposure to PMA blocked the differentiating effects of cholera toxin.

Discussion

Cells of the melanocyte lineage undergo phenotypic changes as they migrate from the neural crest to their final destination in the skin (25, 26). The most striking trait of mature melanocytic cells is the presence of the pigment melanin. The synthesis of melanin is regulated by the specialized cellular enzyme tyrosinase, and the expression of melanin and tyrosinase activity is associated with the

expression of a set of cell surface and melanosomal antigens (6, 23, 27). In earlier studies, we have proposed a model for melanocyte differentiation based on the expression of these traits by a panel of melanoma cell lines and by cultured fetal, newborn, and adult melanocytes (6). The present investigations with cloned melanoma cell lines have extended these studies and have confirmed that the pattern of antigen expression is related to other differentiation traits, such as pigmentation, tyrosinase activity, and morphology. The induction of coordinated changes in the differentiation program of these clones by PMA and cholera toxin was in accordance with predictions from this model.

There are two major stages of melanocyte differentiation in this model—early and late—with an intermediate stage that characterizes the transition from early to late. Melanoma cells at an early stage appear to be committed to the melanocyte phenotype, because they express markers that characterize the melanocyte lineage (6) and they stably maintain this phenotype unless induced to differentiate. The late stage coincides with the emergence of a specialized phenotype, characterized by the appearance of pigment and associated markers. The transition from the early to the mature phenotype is associated with a coordinated change in the pattern of expression of differentiation traits. Even within a stage of differentiation, there appears to be a hierarchy for expression of traits. For instance, as melanocytic cells mature, late antigens do not appear synchronously but rather are expressed in a specific order—C350, then CF21, followed by TA99.

Each of these differentiation traits can be expressed by melanomas in a heterogeneous fashion. For example, it is not unusual to observe a nonpigmented metastasis next to a pigmented metastasis in a patient with melanoma. Likewise, melanocyte differentiation antigens are characteristically expressed by only a subset of melanoma cells within individual lesions (12, 24). These observations have led us to consider whether the remarkable phenotypic heterogeneity observed in melanomas is due to the coexistence of populations of cells at different stages of differentiation. It is clear from our experiments that melanomas are not frozen at a specific stage of differentiation. Appropriate signals, such as phorbol esters, activators of cellular cyclic AMP (e.g., cholera toxin, melanocyte-stimulating hormone) or cyclic AMP analogues can induce melanoma cells to undergo variable degrees of differentiation (27–34). In particular, cyclic AMP has been implicated in the regulation of melanin synthesis in vivo and our experiments suggest that cyclic AMP and other pathways may be involved in the coordinated upregulation and downregulation of a family of traits in melanocytic cells. When melanoma clone 22.a was induced to mature, late markers (melanin synthesis, tyrosinase activity, the melanosomal markers TA99 [gp75] and CF21, and the C350 cell surface glycoprotein [gp180]) were expressed, while in the same cells early and intermediate markers (Ia antigens, M111 [gp110], CALLA [gp100], and the melanoma chondroitin sulfate proteoglycan antigens [mCSP]) were simultaneously downregulated. We have noted that melanoma clones vary in their response to cholera toxin or PMA. The response or lack of response by individual clones to induction must be related to biological traits that differ from one clone to another. One explanation is that intracellular pathways mediating

the differentiation program change as cells proceed through the melanocyte lineage. Alternatively, heterogeneity in expression of receptors or response to these signals is unrelated to the differentiation program.

The regulation of Ia (class II major histocompatibility) antigens by melanoma cells is of particular interest. Ia antigens are not expressed by mature melanocytes (unless induced by gamma interferon [35]), but they are constitutively expressed by a proportion of melanomas (6, 35–37). One explanation for this constitutive expression is that Ia expression is regulated by events occurring during the process of malignant transformation. In support of this hypothesis, recent experiments have demonstrated the induction of Ia by the introduction of ras oncogenes into melanocytes (38). In contrast, serological typing of melanomas has shown that expression of Ia on individual tumors corresponds to stage of melanocyte differentiation (6). Our interpretation of this data is that Ia expression is regulated during melanocyte differentiation, the assumption being that Ia is expressed by precursor cells in the melanocyte lineage. Induction experiments with cloned melanoma cells confirm that Ia can be regulated during differentiation of melanoma cells. For instance, clone 22.a of SK-MEL-23 (Ia^+) downregulated the expression of Ia during transition from an intermediate to a late differentiation program. The two scenarios, transformation and differentiation, are not mutually exclusive. The results of our experiments suggest that both transformation-related events and the differentiation program can regulate Ia expression on melanocytic cells.

The parental melanoma cell lines SK-MEL-131 and SK-MEL-23 each contained a mixture of cell populations that reflected a corresponding diversity present in the original tumor lesions. These different cell populations were apparent in early passage cultures, and could be maintained in parental cell lines even after long-term in vitro culture (unpublished observations). In contrast to the results of the study by Cillo et al. (39), we found that the phenotypes of cloned subpopulations were stable if culture conditions were not manipulated.[2] These observations suggest that, at equilibrium conditions, parental cultures maintain a mixture of phenotypes that correspond to various stages of differentiation. Several factors may have impact on the diversity of populations present within the parental strains: (*a*) small subpopulations in stochastic equilibrium may be competent for only a particular signal or set of signals, and conversely a given signal for differentiation may affect only a small population of cells; (*b*) the degree of variation in the differentiation stage of melanoma cells within a lesion or cell line appears to be limited; thus, in studies with cell lines we have not observed mature melanoma cells mixed with early stage cells, but rather have seen cells at an early stage coexist with intermediate stage, and intermediate cells with late stage cells; and (*c*) within the context of the parental population, induction of differentiation in a subpopulation may produce a decreased growth advantage, selecting against differentiation of the parental culture.

[2] The inability of Cillo et al. to detect a differentiation pattern of antigen expression on melanoma cells is probably due to the limited panel of monoclonal antibodies and cell strains studied. Most of the monoclonal antibodies in this study (e.g., anti–HLA-A,B,C) did not distinguish identifiable subsets (although analysis of their data does suggest that expression of CALLA is inversely related to Ia antigens).

The fact that tumors can be composed of complex populations of cells should be taken into consideration in the evaluation of agents that induce differentiation. Such agents can be used to induce terminal differentiation of tumor cells (40) or to manipulate the expression of antigens on tumor cells, for instance, to optimize antigen expression on lesions in preparation for targeting with monoclonal antibodies. An agent that induces differentiation of cells at one stage may not have the same biological effect on cells at another stage. In addition, agents may interfere with each other in effective induction of differentiation. For instance, we and others have observed that PMA can block the induction of late differentiation markers in melanoma cells (41). Cloned cell lines have definite advantages over parental cell strains in the analysis of agents that induce shifts in the differentiation state. In this regard, neither PMA nor cholera toxin alone or in combination was able to induce major changes of phenotype in 10 noncloned, established melanoma cell lines (Houghton, A. N., and F. X. Real, unpublished observation) including SK-MEL-131 and SK-MEL-23. Cloned subpopulations permitted a dissection of the regulation of differentiation traits in distinct subpopulations. Understanding signals involved at each stage of melanocyte differentiation will lead to strategies aimed at the terminal differentiation of melanoma cells in vivo.

Summary

Phenotypic heterogeneity is a characteristic feature of tumor lesions in patients with melanoma. Variability can be observed in cell morphology, pigmentation, and antigen expression. To test whether phenotypic heterogeneity could be the result of events regulated during cell differentiation, we evaluated the expression of a panel of differentiation traits on melanoma cells. Metastatic melanoma lesions from two patients, designated FD and AP, were examined histologically and found to contain mixed populations of cells. Established melanoma cell lines derived from each of these lesions were subcloned at early passage in culture (passages 7 and 8) to create a panel of clones derived from each tumor. There was heterogeneity in the expression of differentiation-related traits in clones, corresponding to distinct phenotypes observed within the original tumors. Clones from patient FD corresponded to early to intermediate stages of melanocyte differentiation, and clones from patient AP ranged from intermediate to late stages. The influence of cholera toxin and PMA on differentiation of parental cultures and subclones was studied. Results of induction studies demonstrated a number of features of differentiation of melanoma cells: (*a*) regulation of differentiation traits is coordinated as a program of traits expressed sequentially at specific stages; (*b*) early traits, such as the epidermal growth factor receptor and the melanoma chondroitin sulfate proteoglycan antigen, are downregulated as melanoma cells differentiate, whereas late markers, including melanin, tyrosinase activity, and antigens expressed in mature melanosomes, are upregulated; (*c*) Ia (class II major histocompatibility) antigens are characteristically expressed on melanomas corresponding to early or intermediate stages of differentiation and are regulated as part of the differentiation program; (*d*) minimal changes in stage of differentiation were observed during induction of parental cultures with either

cholera toxin or PMA, whereas definite shifts in differentiation could be induced in selected cloned subpopulations. We conclude that melanoma cells are not frozen at a specific stage of differentiation, but rather are capable of differentiating when exposed to appropriate signals. Diversity in the differentiation state of melanoma cells can account for much of the phenotypic heterogeneity observed in melanoma lesions.

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