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Mechanisms of Differentiation in Melanoma Cells and Melanocytes

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Literature is reviewed on the mechanisms of differentiation in mammalian melanoma cells and normal melanocytes. Pigment cells are particularly useful for studies requiring the observation of differentiation in living cells, for example, studies of commitment. Topics discussed include melanin synthesis and other markers of pigment cell differentiation; stochastic models of differentiation and commitment; the lability of early stages of differentiation; extracellular factors affecting pigment cell differentiation, with implications for intracellular controls; the role of proliferation and the cell cycle in differentiation, and the relative roles of changes in transcription, translation, and posttranslational processes.

Introduction: A Model System With Specific Uses

Considered in terms of molecular biology, the study of differentiation in melanoma cells is in its infancy as compared to that of the lymphoid, erythroid, and myogenic lineages in mammals. However, there are two features unique to pigment cells that enable this model system to add its own particular contributions to our understanding of differentiation. One is the production of the polyquinonoid pigment melanin. Evolved for absorption of light, melanin is visible in minute amounts and hence is an ideal marker of differentiation. It can be seen in single cells, which can be alive, unstained, and even at early stages of maturation (*1-5*). This has been particularly beneficial in the study of commitment.

The second feature is the comprehensive background in classical genetics. Coat-color mutations of mice, for example, have been mapped to over 50 loci; many of these act during embryonic development and differentiation, providing a rich source of material for analysis at the cellular and molecular levels (*6*). The cosmetic aspect of human pigmentation and the steadily increasing incidence of malignant melanoma among Caucasian populations (*7*) provide more general reasons for the growth of interest in melanoma and melanogenesis.

Melanoma cells are among the easiest to establish in culture, and a great many human and animal melanoma lines have been derived. For a number of these lines, the induction or promotion of pigmentation by specific agents or conditions has been reported. Some of these lines are listed in Table 1, including the two which together account for the great majority of work on melanoma differentiation. These are the B16 (*8,9*) and Cloudman or S91 (*10,11*) lines, and their subclones, both originally from transplantable melanomas of inbred mice (*8,10*). This review will be concerned mainly with research on such melanoma cells, but will also include some studies of nontumorigenic human and mouse melanocytes, which have recently yielded some informative additions and contrasts.

Normal Melanocyte Lineage

For the discussion of differentiation, some terms will be needed from the biology, biochemistry, and development of normal melanocytes. These fields have been reviewed in detail by Fitzpatrick et al. (*1*), and the biochemistry of melanin synthesis has also been reviewed by Lerch (*12*), Prota (*13*), and recently by Hearing and Jiménez (*14*).

Mature melanocytes are dendritic cells found basally in the epidermis and hair follicles and in some internal organs (*1,6*). The melanin granules that they secrete, and which are endocytosed by neighboring keratinocytes, are actually specialized subcellular organelles called melanosomes, or aggregates of these (*1*). Melanin is synthesized in the melanosomes from the amino acid tyrosine, or tyrosine and cysteine in the red and yellow pheomelanins (*1,12-14*). Most of this reaction pathway can proceed spontaneously, the first step however requiring and the second being accelerated by the melanosomal enzyme

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Table 1. Some melanoma lines in which pigmentation can be induced or stimulated.

Line	Species	Inducing agents or conditions[a]	References
Widely studied			
B16	Mouse (black, C57BL)	Many[b]	(*8,9*)
S91 or Cloudman	Mouse (brown, DBA/2J)	Many[b]	(*10,11*)
Others			
RPMI 3460	Hamster	Theophylline, cAMP, papaverine	(*89*)
19/30 independent melanoma lines	Human	High cell density	(*96*)
HO	Human	TPA and other phorbol esters, DMSO	(*96,97*)
Hs939	Human	Retinoic acid	(*98*)
8/11 melanoma lines	Human	Theophylline, α-MSH, dbcAMP, PGE_1	(*99*)
4 melanoma lines	Human	Mezerein, interferon	(*59*)
SK-MEL-23&131, clones 22a and others	Human	Cholera toxin, TPA	(*100*)
LiBr	Human	Theophylline, DMSO, retinoic acid	(*101*)

[a]Abbreviations: cAMP, cyclic adenosine monophosphate; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; DMSO, dimethyl sulfoxide; α-MSH, α-melanocyte-stimulating hormone; dbcAMP, dibutyryl cAMP; PGE_1, prostaglandin E_1.
[b]See Table 4.

tyrosinase, through its two distinct activities, a monophenolase (EC 1.14.18.1) and a diphenolase activity (EC 1.10.3.1) (*12*). These oxidise tyrosine to dihydroxyphenylalanine (dopa), and dopa to dopaquinone, respectively. The diphenolase activity also accelerates later steps in melanin synthesis, as detailed elsewhere (*12,14*). Evidence has been presented for participation of a second enzyme in this pathway, termed dopachrome oxidoreductase (DCOR), although the purification of this has not been reported to date (*15*).

Melanocytes develop from unpigmented precursor cells, melanoblasts, which originate from the neural crest and migrate through the embryonic dermis to reach their final epidermal sites (*1,16,17*). The definition used here will be that of Hirobe, namely: melanoblasts may have premelanosomes (melanosome precursors lacking melanin), but contain no tyrosinase activity (*18*). This can be ascertained by a histochemical test for tyrosinase, the formation of a brown to black product from L-dopa (*18,19*). There is also an intermediate cell type that can be cultured and cloned from neonatal human skin. These cells are unpigmented by light microscopy, but tyrosinase-positive by the dopa test (*20*). They have been termed premelanocytes, as they can generate pigmented melanocytes in culture (*20*). The equivalent of these cells *in vivo*, if any, is not known, but it may be speculated that they are related to the unpigmented melanocytes in the sheaths of adult human hairs, which apparently show stem cell behavior by regenerating functional melanocytes after skin damage (*21*). Melanocytes found in the same position in mouse hair follicles are suggested to be the source of chemically induced melanotic tumors, another variety of stem-cell behavior (*22*).

Markers and Measures of Pigment Cell Differentiation

It will be helpful at this point to distinguish between differentiation and melanogenesis in pigment cells. Melanogenesis is the biosynthesis of melanin, either by pigmented or by previously unpigmented cells. Differentiation is defined here as the production of different cell types in development (*23*), including the generation of any cell type (e.g., melanocyte) from its precursor (e.g., melanoblast). Definitions of a change in cell type have been discussed previously (*24*). Here it is sufficient to note that differentiation has two features that distinguish it from a simple metabolic adaptation: a) complexity: empirically, precursor cells always differ in a number of ways from their product cells; and b) stability or commitment: the completed change in cell type cannot be reversed under specified conditions, usually removal of the inducing stimulus.

Commitment does occur when melanoma cells and melanocytes become pigmented as discussed below. The pigmentation process is also complex, being associated with a set of other cellular changes such as increased cell size, dendrification (production of dendrites) and alterations in specific protein and mRNA synthesis (Table 2). Typical morphological changes during induced pigmentation of a B16 melanoma subline are shown in Figure 1 as an illustration of the complexity. This kind of melanogenesis thus answers the description of differentiation, although in malignant cells it will not necessarily be normal differentiation.

It will be seen from Table 2 that all the common assays of pigment cell differentiation are assays of melanogenesis. This should be remembered because the two may not always be proportional. For example, an agent that simply increased tyrosine uptake could well increase the rate of melanogenesis but not differentiation. Of course, the same problem can apply to studies of other cell lineages, for example, where erythroid differentiation is measured solely by hemoglobin synthesis. In the case of pigment cells it is to be hoped that changes in transcription and in membrane antigens (Table 2) will soon be more widely useable as independent markers of differentiation.

Only one transcriptional change has been reported so far during melanoma differentiation (Table 2) (*25*). This was detected with a DNA probe selected from a human melanocyte cDNA library with an antityrosinase antiserum. However, the probe is believed to code not for tyrosinase but for an antigenically related protein, possibly the melanocyte glycoprotein gp75 (*26*). Four other recent reports describe cDNA clones selected in similar attempts to clone human or mouse tyrosinase (*27–30*), and three of these are proposed to be authentic sequences for tyrosinase (*27–29*). However, at least one of them appears to code for something else. This is clone pMT4 (*27*), which cross-hybridizes to clone 5A (*30*), and maps at or near the brown (*b*) locus in the mouse (*30*). It is thus unlikely to code for tyrosinase, as no mutation at this locus reduces tyrosinase activity (*6*). Jackson speculates that the product, "tyrosinase-related protein," may be either an enzyme such as DCOR, or a melanosomal structural protein (*30*). Conversely, clones Pmel-34 (human) (*28*) and Tyrs-33 (mouse) (*29*), which are mutually highly homologous (*30*), both map at or near the mouse albino (*c*) locus, at which all mutations reduce tyrosinase activity. Even so, further proof is needed that these do represent tyrosinase, especially because, as with pMT4 (*14*), both predicted proteins (*28,29*) show some significant differences from the reported amino acid composition of mouse tyrosinase, on which five independent studies agree well (*14*).

Stochastic Behavior in Initiation of Differentiation

A stochastic event is just a probabilistic one, with a certain probability either per unit time or following some other event. All molecular interactions are stochastic, for example. So if a cellular process depends on a sufficiently rare molecular event or events, then we may detect the stochastic behavior at the level of the whole cell.

Till et al. proposed in 1964 that the kinetics of commitment of hemopoietic stem cells in mice could be explained by dependence on a single stochastic event (*31*). This was shortly after the first suggestion of a probabilistic step in the cell cycle by Cattaneo et al. (*32*) Since then a variety of workers have proposed stochastic models both for cell differentiation in different mammalian lineages (*33–37*) and for cell proliferation (*38–40*). The idea is appealing because it provides a straightforward mechanism for fine control over the level of proliferation and/or differentiation of a population of stem cells. For example, if a 20% increase in the rate of differentiation is needed, then the probability of differentiation can be raised by that amount through a corresponding increase in the concentration of the putative rare controlling molecule(s) in the cells. Although the response of a single cell will be unpredictable, that of a population will be highly predictable.

Table 2. Cellular changes during pigment cell differentiation.

Marker or property	Direction of change	References	Used as measure?[a]
Correlates of melanogenesis[b]			
Light absorption by melanin	+	(*89,67,102*)	++
Melanosome number/maturity (electron microscopy)	+	(*3,100,103*)	+
Proportion of cells pigmented	+	(*5,41*)	++
Tyrosinase activity	+	(*3,60,95,104,105*)	++
Tyrosinase abundance (immunoprecipitation)[c]	+	(*60,95*)	+
Others widely reported[b]			
Cell size (volume, protein content or area)	+	(*65,105*)	
Dendrification	+	(*86,97,106*)	+
Proliferation rate	− or +[d]	(*3,60,87,88*)	
Reported for mouse melanoma[b]			
Four cellular polypeptides	− and +	(*94*)	
Tumor growth	usually −	(*3,4,105*)	
Hematogenous metastasis	+	(*105*)	
Transcription of Pmel 17-1 mRNA[e]	+	(*25*)	
Reported for human melanomas and melanocytes			
Ganglioside G_{M3}	+	(*97*)	
Antigen Leo Mel 3 (ganglioside G_{D3}?)	+	(*101*)	
Antigens M-4 to M-8	−[f]	(*106,107*)	
Antigens M-9, M-10,, Mel 1	+[f]	(*100,106,107*)	
Glycoprotein gp 75[e]	+	(*26,100*)	
Antigens Ia, AO10, M111, mCSP[g]	−	(*100*)	
EGF receptor	−[f]	(*100*)	
Antigens CF21, C350	+	(*100*)	

[a](+) used as a measure of differentiation occasionally, or newly introduced; (++) often used as a measure.
[b]All changes listed under these headings are inducible by MSHs
[c]Tyrosinase or immunologically related protein(s) (see text).
[d]Dependent on culture conditions (see text).
[e]Transcript Pmel 17-1 is suggested to code for gp 75, thought to be a melanosomal glycoprotein (*26,100*).
[f]Markers correlated with pigmentation among different lines, rather than induced. Markers M111 and CALLA (common acute lymphoblastic leukemia antigen) are reported to correlate with an intermediate level of pigmentation (*100*).
[g]Melanoma chondroitin sulfate proteoglycan.

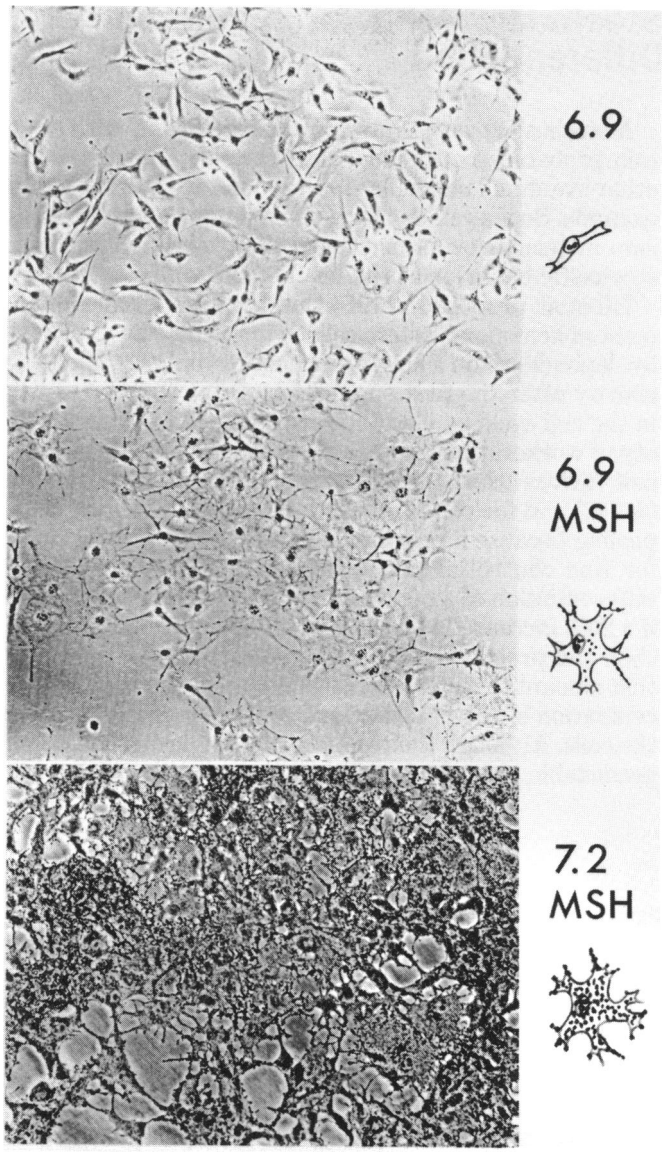

FIGURE 1. Morphology of differentiation in B16F10 melanoma cells. Undifferentiated cells were maintained by frequent subculture at low population density in a supplemented MEM medium (*105*) with 25 mM sodium bicarbonate and 10% CO_2 (equilibrated pH 6.9). Cells (10^4/mL) were plated in this growth medium. After attachment the medium was replaced by (top) the same; (center) as top but supplemented with the stable analog of MSH, [4-norleucine, 7-D-phenylalanine]-α-MSH (1 nM); (bottom) as center but with 45 mM bicarbonate (pH 7.2). Cultures were grown for 4 days, fixed with formalin, and photographed using a 20× phase contrast objective. See Bennett et al. for further details (*105*).

Nonetheless, elegance is no proof, and repeated attempts have been made to test such models.

Melanoma cells were used in one such attempt (*5*). The B16 subline B16C3.6 (*5,41*) was grown under conditions in which nearly all the cells were unpigmented. After transfer to conditions promoting differentiation, the cells were observed continuously by time-lapse cinemicrography. It was thus possible to score the time of first appearance of visible pigment in each cell. This was temporally associated with distinct changes in cell shape, size, and cytoplasmic movements (*5*). There was little or no delay before pigmented cells began to accumulate, showing that melanogenesis became visible under these conditions soon after its actual onset.

Even with freshly cloned cells and conditions giving very rapid pigmentation, this initiation of differentiation occurred at a wide range of different times in individual cells of the same culture (*5*). This heterogeneity of timing could not be ascribed to differences in cellular microenvironment, from analyses of cell position and contacts. Nor was it due to genetic variation, as shown by selection experiments following the design of Gusella et al. (*33*). The lack of a detectable cause and the quantitative distributions of response times were consistent with a stochastic basis for the asynchrony (*5*).

One interesting feature emerged: The timing of initiation of (visible) differentiation was highly correlated in sister cells (products of the same mitosis), with correlation coefficients of up to 0.95. This occurred even when the parental mitosis was before the start of induction of differentiation. Such a correlation is not consistent with a single stochastic event as the cause of all the asynchrony (*40*), but is compatible with another model of stochastic control (*5*) (next section).

Early Instability and Gradual Commitment

An idea about commitment that is widespread in textbooks is that, in development, first, cells (or tissues) become committed or determined, then later they differentiate overtly or, in other words, acquire characteristics or gene products of the new cell type. This idea arose from transplantation experiments on embryos, in which an undifferentiated embryonic tissue would sometimes develop into the correct adult tissue even when moved to the wrong site (*42*). This was a useful concept in its original context, but it has led to a widespread belief that in any kind of cell differentiation, the first thing to happen will be commitment. Commitment tends to be imagined as a rapid event, the switch that sets off the differentiation process. It is this event that is often postulated to be stochastic (*31,33,34,36,37*). However, the following work with pigment cells suggests to the contrary that commitment does not have to be instantaneous, nor to precede the expression of other differentiated properties.

The data were obtained by clonal analysis of B16C3 melanoma cells (*5,24*), and of diploid human melanocytes (*20*). Cells of various degrees of pigmentation, from a partially differentiated population, were plated singly in cloning wells in growth medium and followed by microscopy at intervals to record proliferation and pigmentation.

With the melanoma cells, some cells proliferated to form large clones, virtually all of which were unpigmented, while others failed to grow progressively and in general remained or became pigmented. Some of the large clones grew from unpigmented cells; others, however, came from progenitor cells that were pigmented, their progeny be-

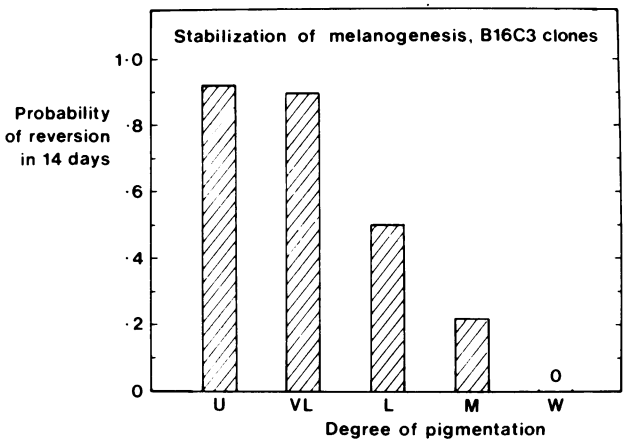

FIGURE 2. Stabilization in melanoma cell differentiation. See text for explanation and Bennett (5) for experimental details. The probability or proportion of cells showing reversion by day 14 is given because reversion was rare after this time. Codes: initial degree of pigmentation of each cell, after overnight attachment; (U) unpigmented; (VL) very lightly; (L) lightly; (M) moderately; and (W) well-pigmented. Grades defined in Bennett (5).

coming unpigmented and resembling the original undifferentiated cells by other criteria (5). In other words, some differentiating cells appeared to revert to the precursor form. Interestingly, the proportion of clones reverting in this way decreased with increasing pigmentation of the progenitor cell (5) (Fig. 2). Reversion was never observed from well-pigmented cells, which could thus be described as fully committed.

Diploid human melanocytes showed an essentially similar pattern, in that some lightly pigmented cells reverted to form unpigmented clones, but there were two notable differences (20) (Table 3). First, diploid pigmented cells could show extensive proliferation, often as quickly as unpigmented cells (premelanocytes) in the same medium. Second, among human melanocytes, reversion to the unpigmented state was rare in cells that were more than very lightly pigmented (Table 3). Some pigmented clones were subcultured and produced up to 10^6 pigmented cells and none unpigmented. This was incidentally a convincing demonstration of commitment with pigmentation, which has since been further extended by the isolation of two immortal, pigmented mouse melanocyte lines (43,44). No unpigmented cells have been detected in the line melan-a in more than 2 years of culture under the described conditions (44).

The reason for the difference in the percentage of clones that proliferated may be trivial. The melanoma cells had been induced to synthesize melanin very rapidly, whereas the melanocytes had developed pigment spontaneously and slowly. Thus, the melanoma cells may have been damaged by the rapid accumulation of melanin precursors, of which at least dihydroxyindole can be toxic to melanoma cells (45). It is more interesting that the potential for reversion was lost earlier in the melanocytes than in the melanoma cells. One cannot generalize about malignancy from one example, but there may be a connection with observations that visibly pigmented melanoma lines in culture are rare (46) and difficult to maintain as such (2).

As a general conclusion, both malignant and normal cells showed gradual commitment, which developed at the same time as pigmentation, and could be quantitated as a falling probability of reversion (e. g., Fig. 2). These observations fit a modified stochastic model (5), as shown in Figure 3. Here the idea of a stochastic initial commitment event is replaced by a stochastic event called initiation, which does switch on differentiation yet which is reversible to begin with. That is, there is initially a finite probability of reversion, which falls with time in the differentiating state. Commitment is complete when this probability reaches zero. The probabilities of initiation and reversion are controlled by extracellular conditions.

The concept of gradual commitment fits the behavior of a wide range of differentiating cell types, and it no longer seems absurd to suggest that it may be more the rule than the exception. It has long been familiar from the simpler organisms Anabaena (47) and Dictyostelium (48). There is now evidence for the activation of differentiated characteristics without or before commitment in most of the principal lineages used to study mammalian differentiation (24), including erythroid cells (49), myoblasts (50), and preadipocytes (51), as well as the early mouse embryo (24,52). Gradual commitment has been described in amphibian embryos (53) and postulated in normal stem cells of the human skin (54), bone marrow and other tissues, in the stem cell continuum model (55).

Extracellular Factors Affecting Melanogenesis and Implications for Intracellular Mechanisms

A good deal of information is available on physiological and pharmacological agents that affect differentiation and melanogenesis of cultured melanoma cells, although there is not much information on factors directly (i.e., in the absence of other cells) affecting differentiation of normal melanocytes (56). Thus it seems most fruitful to concentrate on melanoma cells. Controls over the type of mela-

Table 3. Proliferation and pigmentation of clones from pigmented normal and malignant cells.[a]

Characteristic	B16C3 melanoma	Diploid human melanocytes
Number of clones graded "lightly" to "well"-pigmented[b]	41	23
Number that proliferated by 14 days[c]	12 (29%)	15 (65%)
Number of these still pigmented by 14 days	1 (8%)	13 (87%)

[a]Data pooled from several experiments described in Bennett (5) and Bennett et al. (20). See text for further explanation.
[b]Arbitrary gradations as in Fig. 2 legend.
[c]Defined as clones of three or more cells by day 14; all these subsequently grew progressively, whereas there was considerable variability in the number of days before proliferation began.

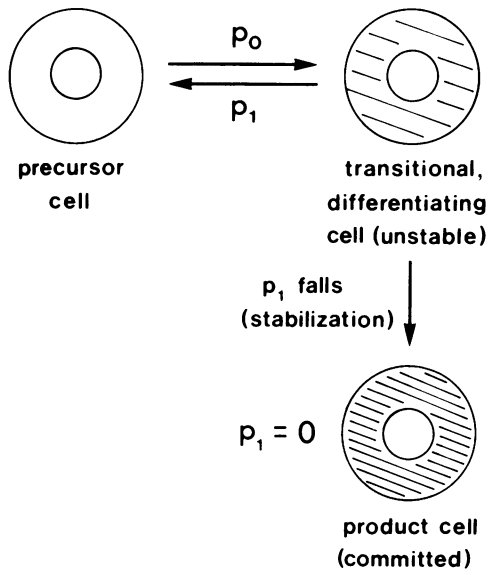

FIGURE 3. A stochastic model of differentiation, incorporating a reversion probability. Figure modified from Bennett (*5*). (p_0) probability of initiation; (p_1) probability of reversion. The fall in p_1 could be due to either the accumulation of a new gene product or the loss of one made by the precursor cell. The probabilities are expressed per unit of time rather than per cell cycle.

nin (eumelanin or pheomelanin) produced by mature mouse melanocytes were reviewed by Takeuchi (*56*).

Agents and conditions known to alter melanogenesis of B16 and S91 cells are listed in Table 4. From reports to date, these two melanomas have shown qualitatively similar responses to all tested agents except retinoic acid (*57,58*). This does not prove that all other pigment cells will behave similarly, because, for example, the phorbol ester TPA, which inhibits pigmentation in both these lines, is reported to promote melanogenesis in human melanomas and in pigmented human melanocytes (*59,60*) while inhibiting it in human premelanocytes (*20*), quail neural crest cells (*61*) and chick melanoblasts (*62*).

It was known at an early stage that cAMP and its analogs promoted pigmentation (*63–65*), and that MSHs increased the intracellular concentration of adenylate cyclase (*66*). This led naturally to the hypothesis that melanogenesis was controlled through cAMP as a second messenger (*3,4*). A number of workers have tested this idea by examining the effects of substances that promote pigmentation upon adenylate cyclase or cAMP in melanoma cells. Table 5 summarizes a selection of these studies. The conclusion is quite clear: Some of these agents increase the level of cAMP and/or its synthesis, but others do not. Thus melanogenesis and/or melanoma differentiation can be altered through both the cAMP pathway and at least one other.

Indeed, from Table 4, one might infer that virtually every major pathway of intracellular signaling impinges on melanogenesis. There is protein kinase C, which tumor promoters like TPA (*67,68*) activate and which would thus presumably inhibit pigmentation in B16 and S91 cells. There is the intracellular pH (*5,69,70*); weak bases have

Table 4. Extracellular factors affecting pigmentation of B16 and S91 cells.[a]

Agent	Effect on B16	Reference	Effect on S91	Reference
Inhibitors of proliferation				
Cytosine arabinoside	+	(*2*)	N	
Colcemid	+	(*2*)	N	
Cyclic nucleotides				
cAMP	+	(*65*)	+	(*63*)
dbcAMP	+	(*65*)	+	(*63,64*)
Methylxanthines				
Caffeine	+	(*65*)	N	
Theophylline	+	(*65,103*)	N	
Isobutyl methylxanthine	+	(*108*)	+	(*109*)
Hormones, vitamins, etc.				
α-MSH and analogs	+	(*67,105*)	+	(*63,110*)
β-MSH	N		+	(*111*)
Adrenocorticotrophic hormone analogs	N		+	(*93*)
Triiodothyronine	−	(*112*)	N	
Pigment-promoting factor	+	(*113*)	N	
Prostaglandins E_1, E_2	N		+	(*88*)
Prostaglandins A_1, D_2	N		−	(*88*)
Retinoids	−	(*57*)	+	(*58*)
1α,25 dihydroxyvitamin D_3	+	(*57*)	N	
Insulin	N		−	(*109*)
Miscellaneous				
High cell density	Biphasic		N	
	or +	(*114*)		
	Biphasic	(*108*)		
	+	(*69*)		
Cholera toxin	+	U	+	(*110*)
Galactose	+	(*115*)	N	
Increased extracellular pH	+	(*5,69,115*)	N	
Weak permeant bases, e.g., imidazole (increased intracellular pH)	+	(*70,71*)	N	
Benzodiazepines	+	(*116*)	N	
Difluoromethyl ornithine (inhibits polyamine synthesis)	+	(*75*)	N	
Ultraviolet light	N		+	(*77*)
TPA and other tumor promoters	−	(*67,68*)	−	(*58*)
Interferon	−	(*68*)	N	

[a]Only representative references are given. (+), (−) promotion and inhibition of pigmentation; (N) no report known to the author; (U) unpublished work in the author's laboratory.

Table 5. Effects of selected promoters of melanogenesis on melanoma adenylate cyclase or cAMP.

Agent	Effect on adenylate cyclase	Effect on [cAMP][a]	Cells	References
MSHs	+	+	S91	(*66,77*)
Adrenocorticotrophic hormone	+		S91	(*66*)
High cell density		0	B16	(*108*)
Isobutyl methylxanthine		+	B16	(*108*)
		+	S91	(*77*)
Theophylline		+	B16	(*4*)
		0	B16	(*117*)
1α,25 dihydroxyvitamin D_3		0	S91	(*57*)
Prostaglandin E_1	+	+	S91	(*66,88*)
Prostaglandin E_2		0	S91	(*88*)
Ultraviolet light		0	S91	(*77*)

[a][cAMP] refers to the intracellular concentration of cAMP; (+) increase in specified parameter; (0) no effect.

been suggested to act by inhibiting lysosomal action (*71*) and specifically MSH receptor turnover (*72*). The effect of pH is not just on tyrosinase, because the stimulatory effect increases (*5*) beyond the pH optimum of tyrosinase, 7.4 (*14*). A role has been postulated for intracellular calcium concentration: methylxanthines affect this as well as cAMP (*73*), while a preliminary study reports stimulation of B16 cell pigmentation by calmodulin and inhibition by its antagonists (*74*). One reason that this would not be surprising is that calmodulin is important in cAMP metabolism. Lastly, the polyamines could play a part, presumably a negative one since inhibition of their synthesis promoted melanogenesis (*75*). Protein kinase C can also promote polyamine synthesis (*76*). It would be of particular interest to elucidate how ultraviolet light, the physiological stimulus of pigmentation in humans, can act directly on melanocytes as has recently been reported (*77*). One possibility is through vitamin D_3 biosynthesis and metabolism (Table 4) (*57*), but it is not yet known whether pigment cells are capable of this. See Fitzpatrick et al. (*1*) for other possibilities. At present it is impossible to say which signaling pathway if any is primary in controlling pigmentation or melanoma differentiation in general.

Differentiation and Cell Proliferation

There is wide interest in possible connections between cell differentiation and proliferation, perhaps especially because in proliferative diseases like cancer, cell differentiation too is usually abnormal. In simpler eukaryotes there are some clear examples of direct connections between differentiation and proliferation. For example, in nematodes, many of the somatic cell divisions are genetically predetermined and give rise to two specified daughter cells of different types and fates (*78*). In budding yeast, the switching of mating type requires the transition between G_1 and S phase known as "start" (*79*). It is still under debate, however, whether any form of mammalian differentiation is directly linked with division, and if so, in what way. Specific models of stem-cell differentiation in relation to division were reviewed previously (*80*). Two general classes of relationship will be discussed here in connection with melanoma cells.

Do Cells Differentiate in a Specific Cell Cycle Phase?

Some authors apparently just assume that differentiation can be initiated only once per cell cycle (*31,33*). Others present indirect evidence that a particular phase such as S phase or G_1 is required for a given form of differentiation (*81,82*); however, others again dispute such evidence (*34,36*), and the author has not encountered any really conclusive example.

There is little published work on melanoma differentiation in relation to the cell cycle. One group suggested that S91 cells respond to cAMP throughout the cycle but to MSH only in G_2 phase (*83*), because MSH receptors are expressed only then (*51*). However, an attempt had been made to synchronize the cells used, and the authors appeared unaware either that such observations can be artefacts due to the synchronization procedure (*84*), or that good synchrony is most unlikely with cells that have such a long doubling time as theirs (*39,40,85*). Moreover, a rapid morphological response to MSH has been reported in 100% of asychronous S91 cells in the presence of cycloheximide, indicating the continuous presence of receptors (*86*).

When growing B16 cells were induced to differentiate with alkaline medium, time-lapse cinemicrography showed a lack of cell cycle dependence (*5*). Initiation of visible pigmentation in individual cells could be at any time from one mitosis to the next; moreover, some cells did not divide at all and so were probably in G_1 phase throughout (*39,40*). Thus, melanoma differentiation in alkaline medium has no relation to the cell-cycle phase, as apparently with cAMP. The possibility of periodic expression of MSH receptors requires further testing.

Is Differentiation Promoted by a Reduced Proliferation Rate?

Many treatments that induce differentiation of cultured cells of numerous lineages also reduce the proliferation rate. This is true of the most commonly used inducers for melanoma cells, including MSH, alkaline pH, and methylxanthines, under standard culture conditions (*4,63,69*). The question thus arises whether differentiation is directly promoted by, or even requires, this reduction.

Most of the answer (for pigment cells) can be seen from Table 6, in which the effects upon proliferation of some inducers and inhibitors of melanogenesis are collated. Clearly, some inducers do not reduce growth; indeed, some are mitogenic under specified conditions. For example, MSH is mitogenic at low tyrosine concentrations or with frequent changes of medium (*87,88*). These conditions would minimize the accumulation of toxic melanin precursors (*45*), suggesting that growth-inhibition can be a result rather than a cause of pigment synthesis. Indeed, several authors report that melanogenesis begins before the reduction of proliferation (*63,70,87,89*). In addition, some inhibitors of differentiation reduce the proliferation rate or do not affect it. In short, reduced proliferation is neither necessary nor sufficient for melanogenesis. It is possible but not proven that reduced proliferation increases melanogenesis in the absence of inhibitory factors.

Nucleus and Cytoplasm: Levels of Control in Melanoma Differentiation

During differentiation there is a coordinated switch from the composition, metabolism, and morphology of the precursor cell to those of the product cell. It is of interest to know whether this set of effects is achieved through changes in rates of RNA transcription, processing or stability, rates of synthesis or posttranslational modification of proteins, or a combination of these. For pigment cell

Table 6. Effects on pigment cell proliferation of selected inducers and inhibitors of melanogenesis.

Agent/condition	Cells	Effect on proliferation[a]	References
Inducers			
MSH, standard medium	S91, B16	0 or −	(*63,67,87,105*)
MSH, "tyrosine-free" medium	S91	+	(*87*)
MSH, serum-free medium	S91	+	(*118*)
dbcAMP, high concentration	S91, B16	−	(*65,119*)
dbcAMP, low concentration	S91	+	(*119*)
Increasing extracellular pH (between 6.9-8.0)	B16	−	(*5,69,105*)
Ultraviolet light	S91, human melanocytes	−	(*77*)
Prostaglandin E_1	S91	−	(*88*)
Retinoic acid	S91	−	(*58*)
Diazepam	B16	0	(*116*)
Cholera toxin	Human melanocytes	+	(*106*)
IBMX	Human melanocytes	+	(*106*)
TPA	Human melanocytes	+	(*106*)
Inhibitors			
TPA	B16, S91	0 or −	(*58,67*)
TPA	Human premelanocytes	+	(*20*)
TPA	Chick melanoblasts	+	(*62*)
Insulin	S91	−	(*109*)
Insulin in presence of MSH	S91	+	(*109*)
Prostaglandins A_1, D_2	S91	−	(*88*)
Triiodothyronine	B16	−	(*112*)

[a](+) increase; (−) decrease; (0) no effect.

differentiation the picture is far from complete, but there is some information.

Recent work with enucleated melanoma cells (cytoplasts) has shown, perhaps surprisingly, that two major aspects of differentiation have a posttranscriptional component. MSH and other inducers can rapidly promote both increased tyrosinase activity (*90*) and dendrification (*86*) of melanoma cytoplasts. Furthermore, neither effect is reduced in intact cells by cycloheximide, in the short term, and both are thus posttranslational; indeed, they are actually accelerated by cycloheximide, suggesting mediation through the loss of a labile repressor (*86,90*). There is no information on whether this may be the same repressor in both cases.

A marked spontaneous activation of tyrosinase was also observed in stored melanoma homogenates (*2,91,92*), with evidence for loss of a repressor in each case (*2,91,92*). There was evidence both for (*91*) and against (*92*) mediation of this loss by a cAMP-dependent protein kinase (PKA). PKA remains a candidate for a mediator of MSH actions. A recent report described the rapid phosphorylation of two cellular proteins in S91 cells exposed to either MSH or dbcAMP (or similar agents) (*93*). Conversely, the rapid dendrification effect mentioned above can be mimicked by an inhibiter of protein kinase C, and suppressed by an activator of it, TPA (*86*). As a provisional interpretation, both activation of PKA and inhibition of PKC may mediate particular actions of MSH.

Not all effects of MSH are posttranslational, however. MSH changes the abundance of certain proteins (*94*), including one that may be tyrosinase as it reacts with an antityrosinase antiserum (*95*) [remembering that there are related proteins that can cross-react with such antisera (*25,30*)]. It is unclear whether these changes in abundance are due to changes in transcription, translation, or degradation, except that MSH does appear to increase the abundance of at least one mRNA, for a tyrosinaselike protein (*25*). If the identities of the latest two putative cDNA sequences for tyrosinase (*28,29*) are confirmed, then no doubt it will shortly be known whether gene transcription for tyrosinase itself is increased in melanoma differentiation.

Concluding Remarks

Pigment cells are being used to study a wide range of aspects of cell differentiation. As mentioned in the introduction, they have been particularly useful in the study of commitment. Nonetheless, and although much has been learned from these and other cells about markers and cellular components that show changes during differentiation, we still understand very little about the crucial questions of how all these changes are initiated, coordinated, and stabilized in a cell. As the future study of these higher level problems is likely to require (among other approaches) both genetics and the use of intact, living cells, we can expect a continued and growing interest in pigment cells as a model system for differentiation.

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