Aging in Epidermal Melanocytes: Cell Cycle Genes and Melanins

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With aging, melanocytes become unevenly distributed in the epidermis. In light skin individuals, hypopigmentation is found in association with focal hyperpigmentation (lentigo senilis). Apparently this results from progressive loss of active melanocytes and focal increase in melanocyte proliferation and/or aggregation. This paper summarizes the present knowledge on aging of melanocytes *in vivo* and *in vitro*, with a focus on the role of melanin as a determinant for proliferation and terminal differentiation. We describe that excessive melanin deposition by cyclic AMP-inducing agents results in increased expression of the cyclin-dependent kinase inhibitors $p27^{Kip-1}$ and $p21^{SDI-1/Waf-1}$, increased binding of p16 to CDK4, and terminal differentiation. Importantly, the efficiency with which the melanocytes exit the cell cycle depends on the melanin background of the donor's cells. Melanocytes from skin types IV–VI that accumulate large amounts of brown black melanin (eumelanin), lose expression of the transcription factors E2F1 and E2F2, two key regulatory proteins, and withdraw from the cell cycle more rapidly than melanocytes from skin types I and II that accumulate red/yellow melanin (pheomelanin). Thus, we propose that terminal differentiation is a tumor suppressor mechanism that becomes less efficient under imperfect eumelanization.
Key words: human melanocytes/differentiation/cell cycle. Journal of Investigative Dermatology Symposium Proceedings 3:36–40, 1998

AGING OF MELANOCYTES IN THE SKIN

Reports from the last four decades have documented that there is a gradual decrease in the number of dyhydroxyphenylalanine-positive melanocytes in the integument of young *versus* old individuals (Szabo, 1954; Staricco and Pinkus, 1957; Snell and Bischnitz, 1963 ; Fitzpatrick *et al*, 1964; Gilchrest *et al*, 1979; Nordlund, 1986). Independent of skin color, the number of α-dyhydroxyphenylalanine-positive melanocytes decreases from 8% to 20% per decade after 30 y of age (Nordlund, 1986; Herzberg and Dinehardt, 1989).

With aging, melanocytes in the skin become a mosaic population manifested by the appearance of large and small melanocytes; the skin becomes mottled and the ability to tan is also reduced (Montagna and Carlisle, 1990). Paradoxically, despite a decrease of active melanocytes, older skin has irregular pigmentation frequently associated with hyperpigmentation. It has been postulated that this heterogeneity in skin pigmentation results from uneven distribution of pigment cells, greater α-dyhydroxyphenilalanine positivity in sun-exposed areas of the skin (Gilchrest *et al*, 1979), a local loss of melanocytes, and a modification in the interaction between melanocytes and keratinocytes (Ortonne, 1990). Solar lentigines, which appears in sun-exposed areas, is considered to be a hallmark of older skin. Electron-microscopic analysis of these lesions has shown an increase in melanocyte number above the dermo–epidermal junction and has also increased melanosomal complexes in surrounding keratinocytes (reviewed by Ber Rahman and Bhawan, 1996). Presently, little or nothing is known about the genesis, proliferative potential, and evolution of these lesions.

Normal pigmentation during wound healing is also affected by age. Older individuals have poorly pigmented, healed wounds that are probably more susceptible to ultraviolet damage (West, 1994). Melanin synthesis in the hair bulb is also lost in senile hair. Apparently, melanocytes are still present in the outer root sheath, but they have lost their differentiated phenotype and became amelanotic (Takada *et al*, 1992). Interestingly, although the number of active melanocytes decreases with age, transplanted human skin from old individuals to immunodeficient mice causes a notable increase in the number of α-dyhydroxyphenilalanine-positive melanocytes in the skin grafts (Gilhar *et al*, 1991). This suggests that in old skin either there are dormant, inactive, melanocytes that under the strong stimuli of an inflammatory response, such as skin transplantation, can become active, or there is a reservoir of nonproliferating melanocytes able to re-enter the cell cycle and actively proliferate. Understanding how old melanocytes respond to grafting could have important implications for re-pigmenting old skin and perhaps for pigmentary disorders such as vitiligo.

It has been demonstrated that monkey choroidal melanocytes, considered to be terminally differentiated melanocytes, have extensive alterations with age in melanosomes, the specialized organelles in which melanin is synthesized and deposited (Hu, 1979). During aging the melanosomes acquire vacuoles filled either completely or partially with homogenous material of low electron density. Some melanosomes fuse together as a melanosome complex enclosed by a unit membrane, and with increasing age the number of melanosomes with alterations increases from 10% to 15% at age 7, to 50% at age 13, and to greater than 60% at age 15. It remains to be determined if similar changes occur in human melanocytes during aging.

Epidemiologic studies have also demonstrated age-related changes in melanocytic nevi. With increased age, there is a progressive decrease in the number of these benign lesions (Ortonne, 1990). Over the course of several years epidermal nevus cells appear to follow a pathway of migration to the dermis and differentiation (Clark *et al*, 1984; Aso

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Abbreviations: α-MSH, α-melanocyte stimulating hormone; cAMP, cyclic adenosine mono-phosphate; CDK, cyclin-dependent kinases; PD, population doublings; SA-β-Gal, senescent-associated pH 6.0 β-galactosidase.

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et al, 1988). Because in the dermis the nevus cells loose the expression of melanocyte markers, it is presently unknown if the nevus cells remain in the dermis or if they are eventually removed by phagocytosis or apoptosis.

AGING OF MELANOCYTES AND NEVUS CELLS *IN VITRO*

Normal human cells in culture are programmed to undergo irreversible functional decrements that mimick age changes in the whole organism (Hayflick, 1979). The limited proliferative potential in culture has been termed replicative senescence. One of the manifestations of replicative senescence that may have profound implications *in vivo* is the loss of the differentiated function of aged cells (Campisi, 1996). Senescent human fibroblasts switch from matrix-producing to matrix-degrading cells: they overexpress collagenase and underexpress collagenase inhibitors, which may contribute to collagen breakdown and thinning of the dermis as observed *in vivo*. In addition, expression of tissue-type plasminogen activator is increased in human senescent fibroblasts (West *et al* 1996). The plasminogen activators are regulators of extracellular proteolysis, therefore overexpression of tissue-type plasminogen activator may also contribute to progressive disruption of extracellular matrix maintenance.

Although enormous progress has been made in understanding the biochemical and molecular mechanisms of pigmentation (reviewed by Hearing, 1998), and pigmentary disorders such as piebaldism (Hosoda *et al*, 1994) and Wardenburg syndrome (Tassabehji *et al*, 1994), the molecular analysis of the life cycle of the normal melanocyte is still in its infancy. We and others have demonstrated that the number of population doublings achieved by human melanocytes exposed to mitogens such as phorbol esters and fibroblast growth factor depends on the age of the donor (Gilchrest *et al*, 1983; Abdel-Malek *et al*, 1994; Medrano *et al*, 1994). Melanocytes from neonatal skin undergo 60–80 population doublings (PD), whereas cells from adults from 20 to 50 y of age proliferate much more slowly and have fewer PD. We have been unable to obtain proliferating cell cultures from donors over 60 y of age. Additionally, melanocytes derived from a Werner's syndrome patient (a premature aging disease) have a dramatically reduced proliferative life-span compared with aged matched controls (2–3 PD *versus* 15–20 PD) (Medrano, unpublished results). This is of particular interest because one of the clinical manifestations of Werner's syndrome is early graying of the hair, mottled pigmentation of the skin, and high frequency of melanomas (Goto *et al*, 1996), thus melanocytes in these patients may be particularly susceptible to mutations. The Werner's gene has been found to codify for a RecQ-type DNA helicase (Yu *et al*, 1996). DNA helicases unwind duplex DNA involved in replication, recombination, repair, and transcription. Chromosomal instability is a characteristic symptom shared by helicase-associated diseases (Ellis, 1996). Therefore, understanding the biologic role of Werner's helicase may be of potential interest for pigment cell biology.

Melanocytes isolated from normal nevi have a greater variability in their *in vitro* life-span compared with normal epidermal melanocytes from age-matched donors (Graeven and Herlyn, 1992; Medrano, unpublished results). Some cultures undergo more than 20 PD, whereas others proliferate well for only 6–10 PD before entering replicative senescence. Hypotheses for such variability include increased genetic instability and/or the presence of different histologic lesions; however, it is likely that the low number of PD attained by some nevi melanocytes may reflect the clonal origin of the cells that might have expanded to have a sizeable number *in vivo*, and therefore had only a limited number of PD to grow *in vitro*, before reaching senescence.

Role of the a-melanotropic hormone and the cyclic adenosine mono-phosphate (cAMP) pathway in melanization and terminal differentiation The development of mouse melanocytes from neural crest stem cells is known to require a cascade of specific growth factors. It has been demonstrated that fibroblast growth factor 2 specifies the melanocyte lineage and that steel factor and endothelin 3 regulate selection and proliferation of melanocyte progenitors. In addition, endothelin 3 stimulates the differentiation of these cells to a mature, pigmented melanocyte. Finally, upregulation of pigmentation is attained by the action of α-melanocyte stimulating hormone (α-MSH) (Murphy *et al*, 1997).

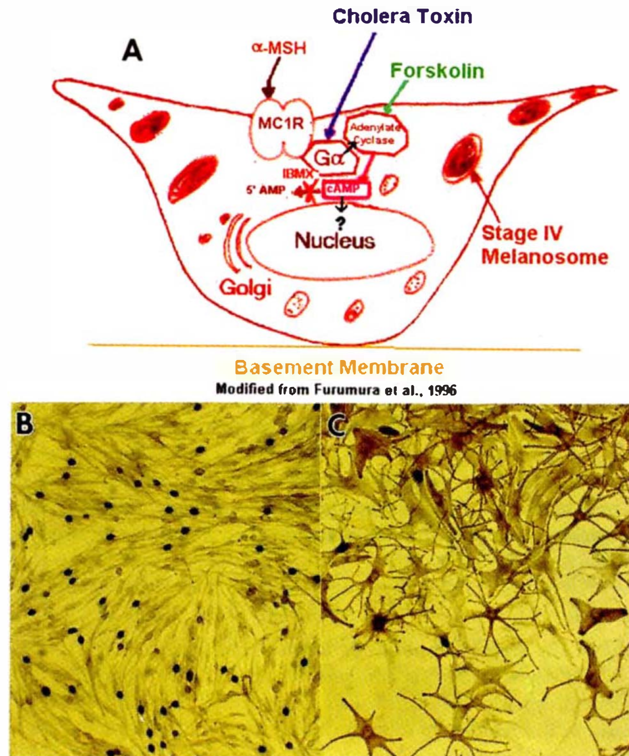

Figure 1. Activation of the cAMP pathway melanization and terminal differentiation. (*A*) The cartoon depicts a simplified cAMP pathway and molecular targets for α-MSH, cholera toxin, and forskolin. (*B*) Incorporation of BrdU in actively proliferating melanocytes. (*C*) Incorporation of BrdU after 4 wk in the differentiating medium. Only a few cells are able to incorporate BrdU in terminally differentiated, pigmented melanocytes. The cells were fixed with 70% ethanol and incubated with anti-BrdU antibody for 30 min at 37°C. After washing cells were incubated with an anti-mouse alkaline phosphatase-conjugated secondary antibody as described by the manufacturer (Boehringer, Indianapolis, IN).

Human epidermal melanocytes also respond to mitogens that target the cAMP pathway, such as α-MSH (Abdel-Malek *et al*, 1995), cholera toxin (Gilchrest *et al*, 1984; Halaban *et al*, 1988; Herlyn *et al*, 1988; Abdel-Malek *et al*, 1992; Medrano *et al*, 1994), forskolin (Haddad and Medrano, unpublished results), dibutyryl-cAMP (Halaban, 1988), and methylxanthines, by increasing melanin production and melanosome aggregation. These agents have different molecular targets in the cAMP pathway, including the MC1R receptor for α-MSH, a Gα protein for cholera toxin, and adenylate cyclase itself in the case of forskolin (**Fig 1A**). Subsequently, the enzyme adenylate cyclase is activated with accumulation of the second messenger cAMP; however, it is still not clear what genes are activated downstream of the cAMP cascade that will eventually result in increased proliferation and melanization.

It is interesting that melanocytes from different skin types show dissimilar rates of proliferation and levels and types of melanin accumulated in response to α-MSH (Hunt *et al*, 1996; Haddad *et al*, 1998). This may be explained in part by the presence of allelic variants of the MC1R that may induce a less potent signaling cascade or even result in complete unresponsiveness to the melanotropic hormone (Hunt *et al*, 1996). MC1R variants with loss of function are found in some individuals with red hair, although red hair can still be found in individuals with a normal MC1R. Unlike α-MSH, cholera toxin does not activate the MC1R (**Fig 1A**), but, as we will demonstrate in this communication, melanocytes exposed to this agent still show a high degree of variability in both proliferation and melanization. This indicates that only a fraction of the very complex pathway of human pigmentation is presently understood and much is left to be discovered.

We have found that in response to cholera toxin, or α-MSH,

melanocytes from skin types IV–VI accumulate high levels of brown/black melanin. This eventually results in loss of the proliferative capacity of the heavily pigmented melanocytes (Abdel-Malek *et al*, 1994; Medrano *et al*, 1994; Haddad and Medrano, unpublished results). **Figure 1(*B*)** shows that, initially, most of the melanocytes respond to the cAMP inducer by increasing their proliferation rates, as shown by their ability to incorporate the thymidine homolog bromo-deoxyuridine (BrdU) into DNA. The continuous culture with cholera toxin, however, results in only a small number of BrdU-positive cells (**Fig 1*C***). Thereby high melanin levels result in loss of the proliferative capacity, when the cells still have potential for many more rounds of cellular division. Operatively, we refer to these quiescent melanocytes as terminally differentiated cells (Medrano *et al*, 1994). Similar results have been obtained with mouse and human melanoma cells after induction of hyperpigmentation by treatment with millimolar concentrations of the amino acid l-tyrosine, a precursor in the melanin synthesis pathway (Guerra *et al*, 1990; Rieber and Rieber, 1994). An early study by Bennett (1983) showed that mouse melanoma cells follow a stochastic model for terminal differentiation after the addition of l-tyrosine, and only the nonpigmented cells continue to proliferate.

EXPRESSION OF CELL CYCLE REGULATORY GENES AND MELANINS

Role of the cyclin-dependent kinase inhibitors The rapid synthesis and degradation of a family of proteins known as cyclins, tightly regulate progression of mammalian cells through the cell cycle. Cyclins constitute the regulatory subunit of another family of proteins known as the cyclin-dependent kinases (CDK). Recently, it has been demonstrated that the activity of such kinases can be negatively regulated by proteins generically known as the cyclin-dependent kinase inhibitors (CDK-I) of which several have been identified (*p21*$^{Waf-1/SDI-1}$, *p27*$^{KIP-1}$, *p57*, *p16*^{INK4}, and *p15*).

In normal cells, the CDK-I's function is to regulate growth, differentiation, and senescence of cells from diverse origin, including fibroblasts and keratinocytes (Hara *et al*, 1996; Hauser *et al*, 1997). In particular, p16 is deleted or mutated in hereditary familial melanomas and other cancers *in vivo* (reviewed by Sherr and Roberts, 1995). The role of the CDK-I in melanocyte biology is complex and still not well understood. An example of this is the unexpected expression of p21 and p27 found in primary invasive melanomas (Breslow thickness greater than 0.076 mm), compared with noninvasive tumors and benign nevi (Maelandsmo *et al*, 1996; Trotter *et al*, 1997; Plowden, Orengo, and Medrano, unpublished results). The presence of high levels of p27 and p21 may confer the invasive melanoma cells' slow growth and resistance to conventional cancer treatments such as chemotherapy and radiation.

We have found that accumulation of high levels of brown/black melanin (eumelanin), after prolonged treatment with cAMP inducers, correlates with increased expression of the CDK-I p27^{Kip1}, poor or absent CDK-2 activity, increased binding of the CDK-I p16 to CDK4, and a modest increase in p21$^{SDI-1/Waf-1}$ levels (Haddad and Medrano, unpublished results).

Analysis of senescent and terminally differentiated melanocytes has revealed common, as well as distinct, alterations in gene expression. Senescent melanocytes have increased p21 and p16 levels but not p27, whereas terminally differentiated melanocytes have a large increase in p27 levels, a modest increase in p21, and no changes in p16 (**Fig 2*A–C***). Terminally differentiated and senescent melanocytes, however, have an identical inability to activate and translocate the kinase ERK2 to the nucleus, compared with young melanocytes cultures (Medrano *et al*, 1994). Studies in progress aim to resolve whether terminal differentiation results in an early senescent phenotype in epidermal melanocytes.

Transcription factors of the E2F family and melanins The E2F consist of five distinct E2F members and at least two heterodimer partners, DP1 and DP2. The individual family members are able to form a variety of protein complexes and may play distinct roles in proliferation, cell cycle exit, and terminal differentiation. E2F1, E2F2, and E2F3 can efficiently activate DNA synthesis in quiescent fibroblasts, whereas E2F4 and E2F5 have little or no activity in S phase induction (DeGregori *et al*, 1997). Changes in E2F DNA-binding activity are detected during erythroid, adipocyte, muscle, and epithelial differentiation (reviewed by Sidle *et al*, 1996). E2F4 and E2F5 form complexes with the p107 and p130 retinoblastoma family proteins and function as transcriptional repressors in quiescent cells (Smith *et al*, 1996). In particular, E2F-p130 complexes may repress E2F1 and E2F2 genes by binding to E2F DNA-binding elements in their own promoters.

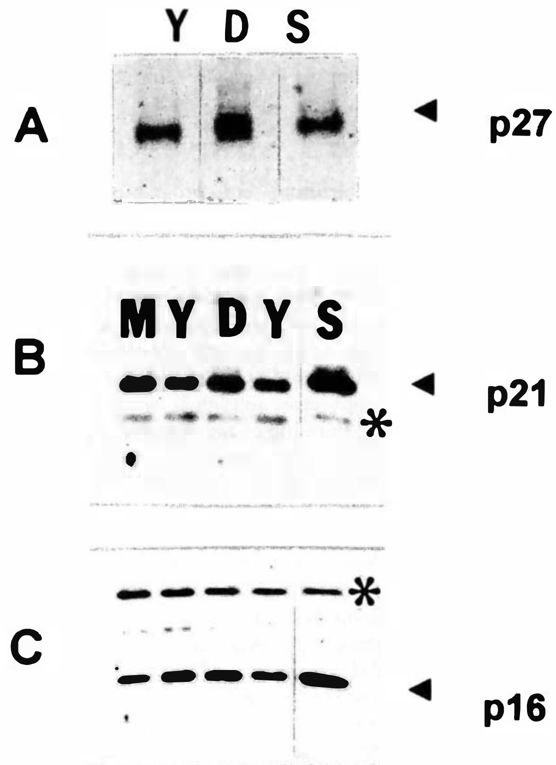

Figure 2. p27^{Kip-1}, p21$^{Waf-1/SDI-1}$, and p16^{INK4} expression in proliferating, terminally differentiated, and senescent melanocytes. Equal amounts (30 μg) of whole cell lysates were electrophoresed in 12% sodium dodecyl sulfate polyacrylamide gel slabs and blotted on nitrocellulose membranes. Membranes were incubated overnight at 4°C in either of the following antibodies: 1:300 dilution of a rabbit anti-p16 (PharMingen, CA); 1:100 mouse anti-p21 (Oncogene Research Products, MA); 1:2000 dilution of a monoclonal anti-p27 (Transduction Laboratories), all in T-TBS buffer. After washing with T-TBS buffer, the membranes were incubated with a donkey anti-rabbit, or a sheep anti-mouse antibody, conjugated to horseradish peroxidase (1:3000). The target proteins were detected using the ECL western blotting detection kit (Amersham, IL). *Unspecific bands recognized by the p21 and p16 antibodies. These bands are shown to demonstrate equal protein loading in the gel. M, primary melanoma cells; Y, young proliferating melanocytes; D, terminally differentiated, nonproliferating melanocytes; S, senescent melanocytes.

We have found that the efficiency with which the melanocytes exit the cell cycle depends on the melanin background of the donor's cells (Haddad and Medrano, unpublished results). After continued exposure to cAMP inducers such as cholera toxin or α-MSH, melanocytes from skin types IV–VI accumulate large amounts of melanin compared with melanocytes from skin types I and II (**Fig 3*A***). This eventually results in reduced or absent expression of E2F-1 (**Fig 3*B*, Mel IV**, *arrowheads*) and E2F2, increased levels of E2F5, and cell cycle exit (Haddad *et al*, 1998). Under the same treatment, however, melanocytes cultured from very light skin show high levels of E2F1 (**Fig 3*B*, Mel I**) and E2F2, and continue to proliferate for at least 6–10 wk (Haddad and Medrano, unpublished results). Current experiments are aimed at determining how the melanocyte's cell cycle machinery senses and responds to melanin accumulation.

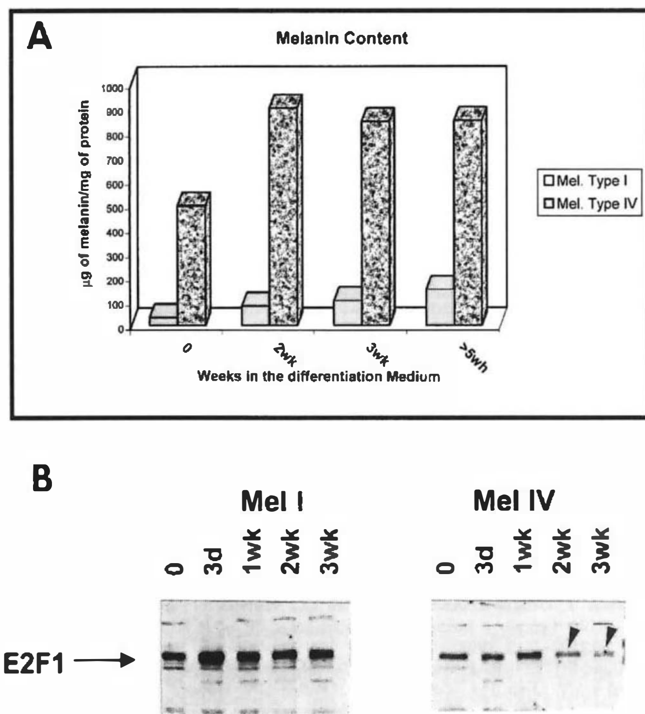

Figure 3. Accumulation of brown/black melanin correlates with reduced E2F1 expression. (*A*) Kinetics of melanin accumulation in naïve melanocytes exposed to a medium containing 10 nM cholera toxin. (*B*) Kinetics of E2F1 expression as determined by western blotting. *Arrowheads* show the decline in E2F1 levels after 2 wk in the differentiating medium, which correlate with melanin accumulation and the onset of terminal differentiation. Mel I, melanocytes were arbitrarily assigned to this group when they did not accumulate appreciable amounts of brown/black melanin and continued to proliferate in the differentiating medium for at least 6 wk. Mel IV, melanocytes from skin types IV–VI.

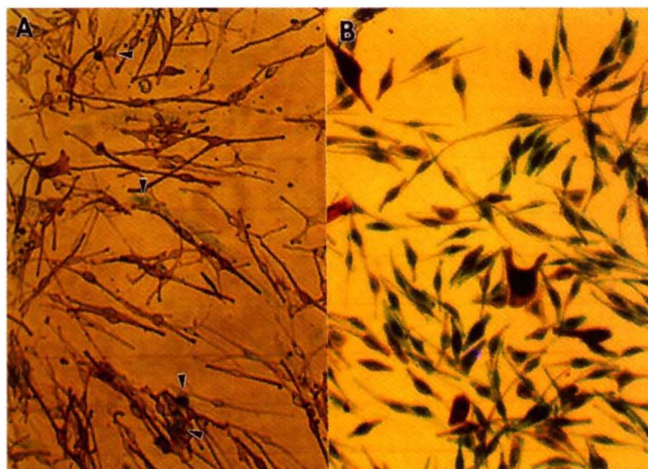

Figure 4. Senescence-associated b-galactosidase (b-Gal) in proliferating and terminally differentiated melanocytes. (*A*) Proliferating melanocytes, only 3–4 cells are stained (*arrowheads*). (*B*) Terminally differentiated melanocytes, most of the cells show intense staining. β-Galactosidase staining was performed as described by Dimri *et al* (1995).

GETTING THE "BLUES" AT THE END OF THE PROLIFERATIVE LIFE-SPAN: CHROMATIN CHANGES, HISTONE ACETYLATION, AND WHAT ELSE?

A biomarker for senescent cells, a pH 6.0 β-galactosidase (SA-β-Gal), has been found to be expressed in senescent fibroblasts *in vitro* and in the human skin of old individuals, and possibly reflects changes in cell function that accompany senescence (Dimri *et al*, 1995). We have found that, whereas in the proliferating population of melanocytes only a few cells are SA-β-Gal positive (**Fig 4*A***), terminally differentiated melanocytes have high levels of this enzyme (**Fig 4*B***). This differs from the original observation that terminally differentiated cells such as keratinocytes do not express SA-β-Gal, although they do become positive when entering replicative senescence (Dimri *et al*, 1995). SA-β-Gal, however, is also detected in fibroblasts induced to irreversibly withdraw from the cell cycle by treatment with histone deacetylase inhibitors such as sodium butyrate and trichostatin A (Ogryzko *et al*, 1996). Therefore it may serve as a marker for various states of terminal loss of proliferation in addition to replicative senescence.

Recent studies have implicated a role for histone acetylation and chromatin structure in growth regulation (reviewed by Wade and Wolffe, 1997). If expression of SA-β-Gal is a common manifestation of such changes in aging, then repressive chromatin domains may be strong determinants for the loss of the proliferative capacity of terminally differentiated melanocytes. The protein p300/CBP, a novel class of acetyltransferase, is a chromatin-remodeling factor. p300 associates with several transcription factors, including the cAMP-responsive element binding protein CREB, a transcription factor known to mediate the response of hormones that activate the cAMP pathway (Ogryzko *et al*, 1996) and with functional RNA complexes (Nakajima *et al*, 1997). In addition, p300/CBP binds to the tumor suppressor p53 (Gu and Roeder, 1997) and stimulates its sequence specific DNA-binding activity. Therefore, p300/CBP appears to be a master regulator of cell function. It will be interesting to determine whether p300/CBP is involved in the cAMP/melanin pathway that leads to melanocyte terminal differentiation.

CONCLUSION

Multiple diverse and redundant molecular changes characterize senescence in mammalian cells (Smith and Pereira-Smith, 1996; Campisi, 1996). A major challenge will be to decipher which ones cause senescence and which ones result from senescence. It is presently unknown if mitotically competent melanocytes exist in old human skin. Moreover, it is still not known how melanocytes from young donors respond to stimuli such as solar radiation and what is the fate of these cells. Previous studies aimed at determining rates of proliferation of UV-exposed melanocytes are plagued by artifacts resulting from considering thymidine incorporation a manifestation of DNA synthesis and not DNA repair; however, because normal, nonsun-exposed skin melanocytes are usually in a nonproliferative state, it is difficult to hypothesize that melanocytes can exhaust their proliferation capacity during the normal life-span of the individual. But, in order to develop strategies for intervention therapies for improving the human pigment cells in old skin it will be necessary to first understand how melanocytes age in the skin.

After exposure to solar radiation or ultraviolet B light, keratinocytes and melanocytes synthesize and release α-MSH (Farooqui *et al*, 1993; Chakarborty *et al*, 1995; Luger *et al*, 1997). Because variants of the MC1 Receptor have been identified as a risk factor for melanoma susceptibility (Valverde *et al*, 1996), we hypothesize that delayed terminal differentiation in melanocytes unable to accumulate brown/black melanin may result in excessive proliferation, contribute to the formation of solar lentigines, and increase the risk of immortalization of these cells.

We propose that terminal differentiation is a tumor suppressor mechanism only partially operative under imperfect eumelanization. Presently it is not known if a similar pathway operates *in vivo*, but heterogeneity in skin pigmentation and in particular the presence of lentigo senilis with aging, and increased frequency of melanomas, in individuals from light skin backgrounds, suggest that this might be the case.

Note added in proof: After submitting this manuscript, Bertolotto *et al.* (*Mol and Cell Biol* 18; 694–702, 1998) reported that cAMP increases micropthalmia (MITF) expression and binding to tyrosine-related protein 1 (TRP1) and TRP2 promoters.

This work was supported by grants AG3663 and AG00594 from the National Institutes of Health. M.H. was supported by a Training Grant from the National Institute on Aging (T32-AG00183).

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