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Invited Review

Structure and function of melanocytes: Microscopic morphology and cell biology of mouse melanocytes in the epidermis and hair follicle

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Summary. Melanocytes characterized by their tyrosinase activity, melanosomes and dendrites locate in the basal layer of epidermis and hair bulb in the skin of mice. Melanocytes differentiate from undifferentiated melanoblasts derived from embryonic neural crest. Melanocyte-stimulating hormone plays an important role in the regulation of the differentiation of mouse melanocytes in the epidermis and hair bulb by inducing tyrosinase activity, melanosome formation, transfer of melanosomes and increased dendritogenesis. The proliferative activity of differentiating epidermal melanocytes of newborn mice during the healing of skin wounds is regulated by semidominant genes, suggesting that the genes are involved in regulating the proliferative activity of epidermal melanocytes during differentiation. The morphology and differentiated functions of mouse melanocytes are shown to be influenced by environmental factors such as ultraviolet and ionizing radiations. From the results of serum-free culture of mouse epidermal melanoblasts, basic fibroblast growth factor is shown to stimulate the sustained proliferation of melanoblasts in the presence of dibutyryl adenosine 3',5'-cyclic monophosphate and keratinocytes. In contrast, melanocyte differentiation in serum-free culture is induced by melanocyte-stimulating hormone in the presence of keratinocytes. These results suggest that the structure and function of mouse melanocytes in the epidermis and hair bulb are controlled by both genetic factors and local tissue environment, such as hormones and growth factors.

Key words: Melanoblast, Melanocyte, Epidermis, Hair follicle, Neural crest

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Introduction

Melanocytes are neural crest-derived cells which synthesize melanin pigments by tyrosinase activity. Fully differentiated melanocytes, characterized by their tyrosinase activity, mature melanosomes and dendrites, can be seen mainly in the hair bulbs of the skin of adult mice, where they secrete melanosomes into the surrounding keratinocytes, giving rise to melanized hairs (Fig. 1). These melanocytes are derived from melanoblasts, undifferentiated precursors, located in the epidermis. Tyrosinase initiates melanin synthesis by catalyzing the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (dopa) and the oxidation of dopa to dopaquinone (Hearing and Tsukamoto, 1991). Recently, other regulatory proteins, known as tyrosinase-related protein-1 (TRP-1, Jackson et al., 1990) and -2 (TRP-2, Jackson et al., 1992) have also been identified. TRP-1 maps to the mouse brown locus (Jackson et al., 1990). In contrast, TRP-2 maps to the mouse slaty locus (Jackson et al., 1992). Recently, TRP-2 has been shown to possess dopachrome tautomerase activity (Tsukamoto et al., 1992) which converts dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (Hearing and Tsukamoto, 1991). The function of TRP-1 remains unclear. Melanin synthesis occurs in specialized organelles called melanosomes (Seiji et al., 1963). Melanosome maturation is categorized in four stages: namely, stage I and II include unmelanized immature premelanosomes, while melanized melanosomes are classified as stage III and IV (Fitzpatrick et al., 1969). It is known that physiological colour changes in fish and amphibians are regulated by the aggregation and dispersion of melanosomes in the melanophores. However, in mammals, skin colours or coat colours are regulated by melanosome transfer from melanocytes to neighbouring keratinocytes and/or to cells of the hair follicles. Mammalian follicular melanocytes produce two types of melanin: brownish-black eumelanin and

reddish-yellow pheomelanin (Prota, 1980). Although differences in molecular size and general properties exist, these melanins arise from a common metabolic pathway in which dopaquinone is a key intermediate (Hearing and Tsukamoto, 1991).

In the mouse embryo, melanoblasts, precursors of melanocytes, originate from the neural crest, migrate lateroventrally from 8 days, and reach all body regions by 12 days of gestation (Rawles, 1947). Recent study has shown that TRP-2 probe detected migratory melanoblasts or neural crest cells as early as 10 days of gestation (Steel et al., 1992). Melanoblasts may invade the epidermis from the dermis between 11 and 12 days of gestation (Mayer, 1973). By 13 or 14 days of gestation, melanoblasts have finished colonization in the epidermis (Mayer, 1973). Mouse epidermal melanoblasts begin to differentiate into active melanocytes at 16 days of gestation (Hirobe, 1984a). Epidermal melanocytes increase dramatically in number after birth (Quevedo et al., 1966; Takeuchi, 1968; Weiss and Zelickson, 1975; Hirobe and Takeuchi, 1977a, 1978); these melanocytes migrate into hair bulbs, giving rise to melanized hairs (Fig. 1, Mann, 1962; Hirobe, 1984a). Melanosomes are produced in varying sizes, numbers and densities in hair bulb melanocytes. The melanosomes are then passed on to hair shaft in hair bulbs, where the final distribution patterns of the pigment are determined. This distribution plays an important role in determining coat colour of mice (Silvers, 1979). The process of melanoblast proliferation and differentiation as well as melanosome transfer are regulated by numerous genes. In mice, more than 150 alleles at over 60 loci are known to affect coat colour phenotypes (Silvers, 1979). Recent advances in molecular techniques have permitted the isolation and sequencing of several of these genes and the molecular basis of the corresponding mutations has been clarified (Jackson et al., 1994). In this article, studies of the structure and function (proliferation, differentiation and interaction with keratinocyte) of epidermal and hair follicular melanocytes in mice in normal circumstances or in experimental conditions by external stimuli, such as wound healing, ultraviolet (UV) and ionizing radiations are reviewed and discussed.

Morphology of mouse melanocytes

Morphology of melanocyte in the epidermis

The epidermis is a stratified squamous epithelium consisting mainly of cells with two different origins: keratinocytes and melanocytes. Keratinocytes comprise the bulk of the epithelium, undergo keratinization and form the dead superficial layers of the skin. These superficial keratinized cells continuously desquamate from the surface and are replaced by cells derived from mitotic cells in the lowest layer (basal layer). The cells are displaced to successively higher levels by the population of new cells below them. As they move upward, they elaborate keratin and accumulate in the cytoplasm (spinous layer and granular layer), and finally almost all cells are occupied by keratin (cornified layer). Melanocytes are cells in the basal layers that do not keratinize but which are capable of producing melanin pigment (Fig. 2). Most of these melanocytes migrate from the epidermis to the hair follicles, and colonize hair bulb melanocytes in hairy general body (trunk) skin. In the glabrous skin of ear, nose, foot and tail, numerous differentiated melanocytes are found in the epidermis of

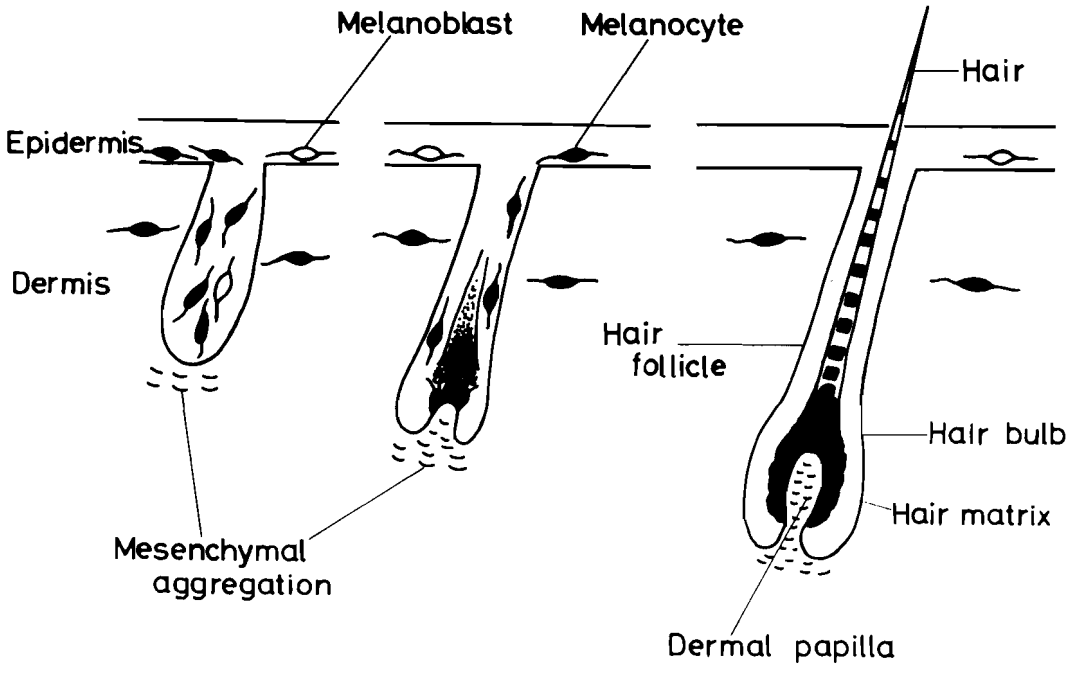

Fig. 1. The developmental stages of mouse hair follicle. Each hair is formed in a follicle which arises as an epidermal invagination. The dermis forms a thickening beneath, and the end of the epidermal invagination comes to surround it. The dermal thickening develops into the dermal papilla. Subsequently, the basis of the follicle thickens, forming a cylinder on the top of the dermal papilla. Undifferentiated melanoblasts and differentiated melanocytes migrate from the epidermis to the hair follicle and colonize the hair matrix. Hair bulb melanocytes secrete mature melanosomes into keratinocytes consisting of hair bulb and as a result hair develops.

adult mice. However, in the epidermis of hairy skin, epidermal melanocytes are only found during the early weeks after birth (Quevedo et al., 1966; Takeuchi, 1968; Weiss and Zelickson, 1975; Hirobe and Takeuchi, 1977a).

The time of onset of the differentiation of epidermal melanocytes in the dorsal skin of C57BL/10J(black) mice has been investigated by histochemical methods, the dopa reaction and the combined dopa-premelanin reaction (Hirobe and Takeuchi, 1977a). The combined dopa-premelanin reaction (combined dopa-ammoniacal silver nitrate staining) reveals undifferentiated melanoblasts that contain unmelanized stage I and II melanosomes in addition to differentiated melanocytes (Mishima, 1960, 1964; Hirobe, 1982b). Cells positive to the combined dopa-premelanin reaction (melanoblast-melanocyte population) were first identified on the 14th day and increased in number until the 17th day of gestation. The population remained constant (ca 140 cells/0.1 mm^2) until 4 days after birth, and then decreased (Hirobe, 1984a). On the other hand, the dopa reaction reveals differentiated melanocytes that contain tyrosinase activity (Hirobe, 1982a, 1984b). Dopa-positive cells were first identified on the 16th day of gestation and increased until 4 days after birth, then gradually decreased and disappeared by 30 days (Hirobe, 1984a). Despite the similarity of the pattern of melanocyte differentiation in different strains of mice, there are dramatic differences in the number of epidermal melanoblasts or melanocytes in different strains of mice (Quevedo et al., 1966; Takeuchi, 1968; Hirobe, 1982a, 1984b, 1985, 1987, 1992b; Tamate et al., 1986, 1989).

The ultrastructural morphology of mouse epidermal melanocytes has been studied by electron microscopic observations. Weiss and Zelickson (1975) reported that melanocytes containing melanosomes of several developmental stages were present in the epidermis of 15-day-old C57BL/6 mouses embryo and subsequently increased in number. Numerous melanocytes containing all stages of melanosomes were present until 3 days after birth, and then they declined in number (Weiss and Zelickson, 1975). In C57BL/10J mouse, melanoblasts containing unmelanized stage I melanosomes (Fig. 3A) gradually decreased in number after birth, while the number of melanocytes containing melanized stage III and IV melanosomes (Fig. 3B) rapidly increased (Hirobe and Takeuchi, 1978). In 6-day-old mice, epidermis contained melanocytes with numerous stage IV melanosomes and with no or only a few melanoblasts (Hirobe and Takeuchi, 1978). In addition, Golgi apparatus and rough endoplasmic reticulum (RER) decreased in number during the differentiation of epidermal melanocytes, though the amount of smooth endoplasmic reticulum (SER) and mitochondria showed no notable change (Hirobe and Takeuchi, 1978).

The process of melanosome formation in the differentiating melanocytes in the epidermis of newborn C57BL/10JHir mice has been studied by using an electron microscope (Hirobe, 1982b). Numerous stage I melanosomes were observed around the Golgi vacuoles. Direct continuity between stage I and II melanosomes

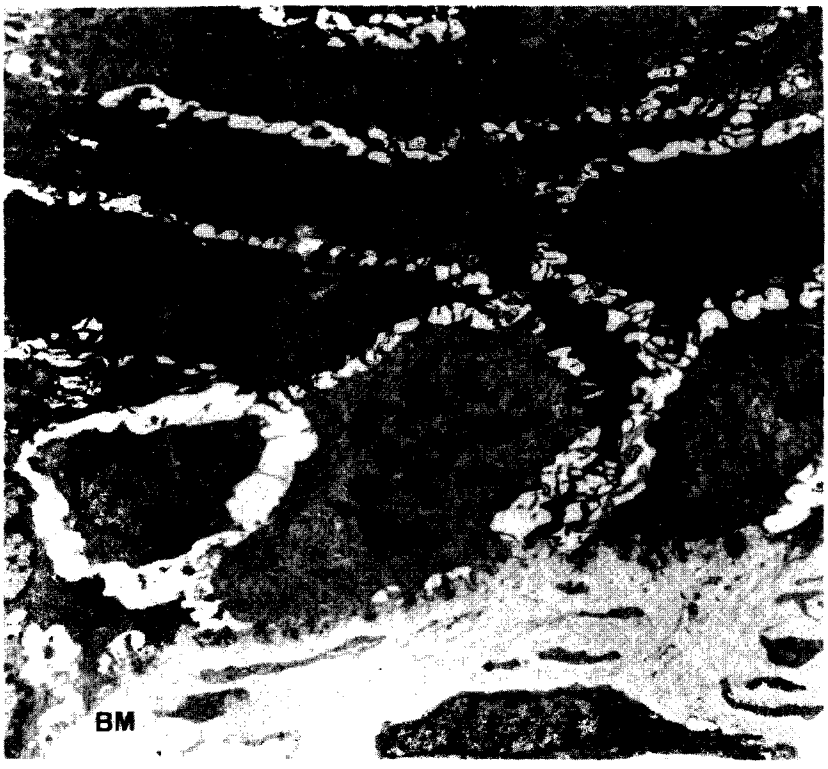

Fig. 2. Electron micrograph of the epidermis of a 3-day-old C57BL/10J mouse. An epidermal melanocyte (M) resides in the basal layer (the lowest layer) of epidermis and contacts with basal keratinocyte (K) through small processes. Basal keratinocytes sit on the basement membrane (BM). Epidermis is a stratified squamous epithelium. On the top of the basal layer, the spinous layer (S) is observed. The granular layer (G) which possesses well-developed keratohyalin granules (KG) sits on the top of the spinous layer. KM: mitotic keratinocyte; F: fibroblast; T: tonofilament. x 5,600

and RER was also observed. Moreover, various stage III and IV melanosomes were located close to the RER. All stages of melanosomes were posotive to ammoniacal silver nitrate reaction. However, Golgi apparatus, RER and vesicles were negative. In contrast, stage I and II melanosomes were dopa-negative, whereas stage III and

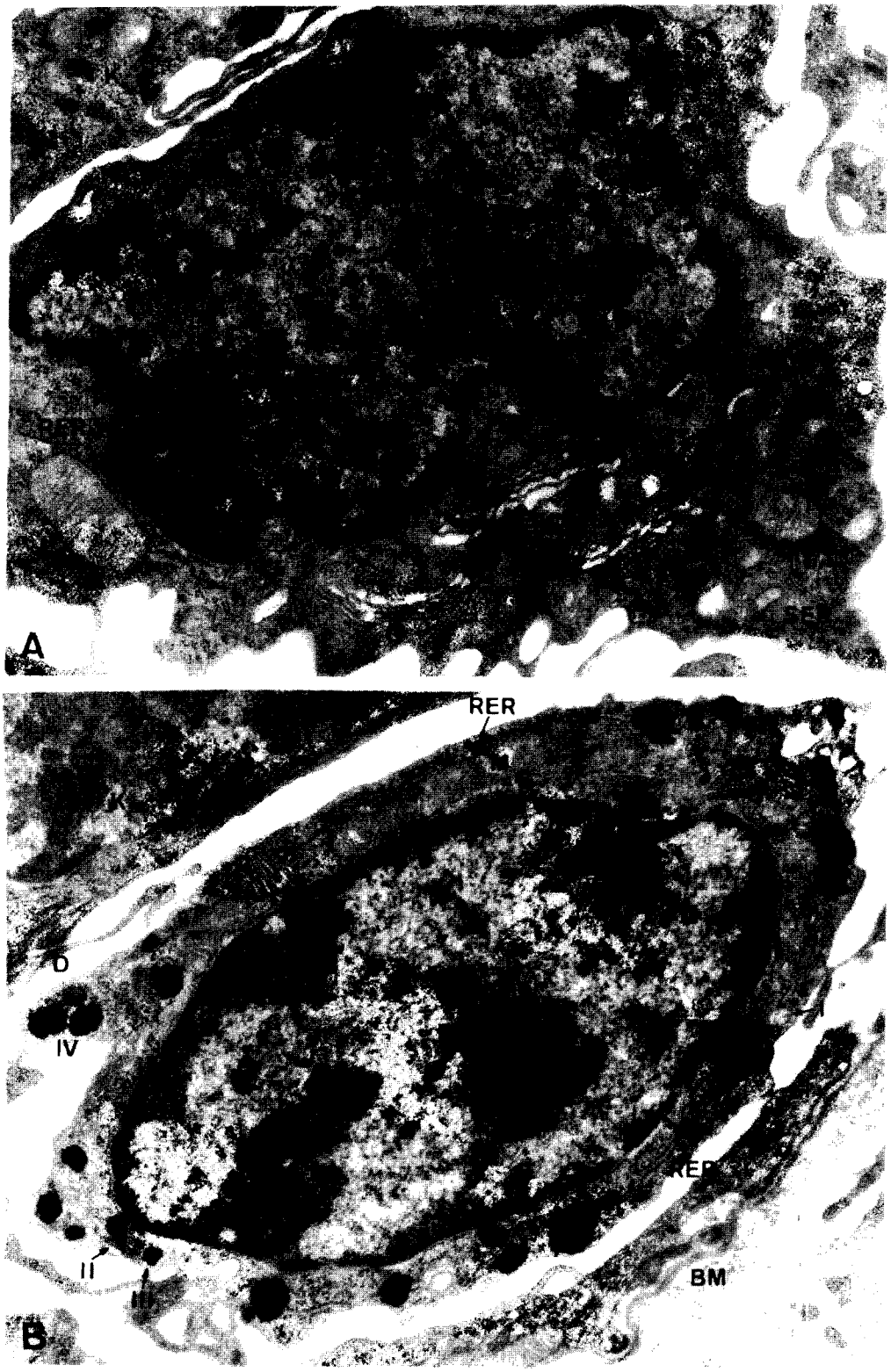

Fig. 3. A. Electron micrograph of an epidermal melanoblast of a 1-day-old C57BL/10J mouse. Numerous stage I melanosomes (arrows) are seen around the well-developed Golgi apparatus (G). RER: rough endoplasmic reticulum; SER: smooth endoplasmic reticulum; M: mitochondrion; K: keratinocyte. **B.** Electron micrograph of an epidermal melanocyte of a 3-day-old C57BL/10J mouse. Numerous stage IV melanosomes (IV) are seen. I: stage I melanosome; II: stage II melanosome; III: stage III melanosome. G: Golgi apparatus; RER: rough endoplasmic reticulum; M: mitochondrion; K: keratinocyte; BM: basement membrane; D: desmosome. A, x 24,000; B, x 26,800

IV melanosomes were dopa-positive. Dopa-melanin depositions were also observed in trans-Golgi saccules and small vesicles derived from these saccules but not in RER. These vesicles were in contact with, or fused to, melanosomes (Hirobe, 1982b). These findings suggest that tyrosinase may be transferred by Golgi vesicles into stage I and II melanosomes originating from Golgi vacuoles or RER, and then stage III and IV melanosomes are developed.

Morphology of melanocytes in the hair follicles

The hair is formed in the hair follicle which begins as an epidermal invagination into the dermis. The dermis forms a thickening beneath, and the end of the invagination comes to surround it. The dermal thickening develops into the dermal papilla, and the surrounding part of the invagination forms the hair bulb (Fig. 1). The hair follicular melanocytes derived from epidermal melanocytes are highly dendritic cells and colonize the hair matrix, lower half of the hair bulb. Hair bulb melanocytes secrete stage IV melanosomes into keratinocytes consisting of hair bulb and as a result hair cortex and medulla develops. In mice, the process of morphogenesis of hair follicle structures is cyclic (Dry, 1926) and called hair growth cycle (Chase, 1954). The hair growth cycle consists of three stages: resting (telogen); growth (anagen); and regression (catagen). Anagen hair follicles develop to produce hair shafts formed by pigmented melanocytes and fully keratinized cells (Dry, 1926; Chase, 1954). This deposition of stage IV melanosomes continues during the entire growth

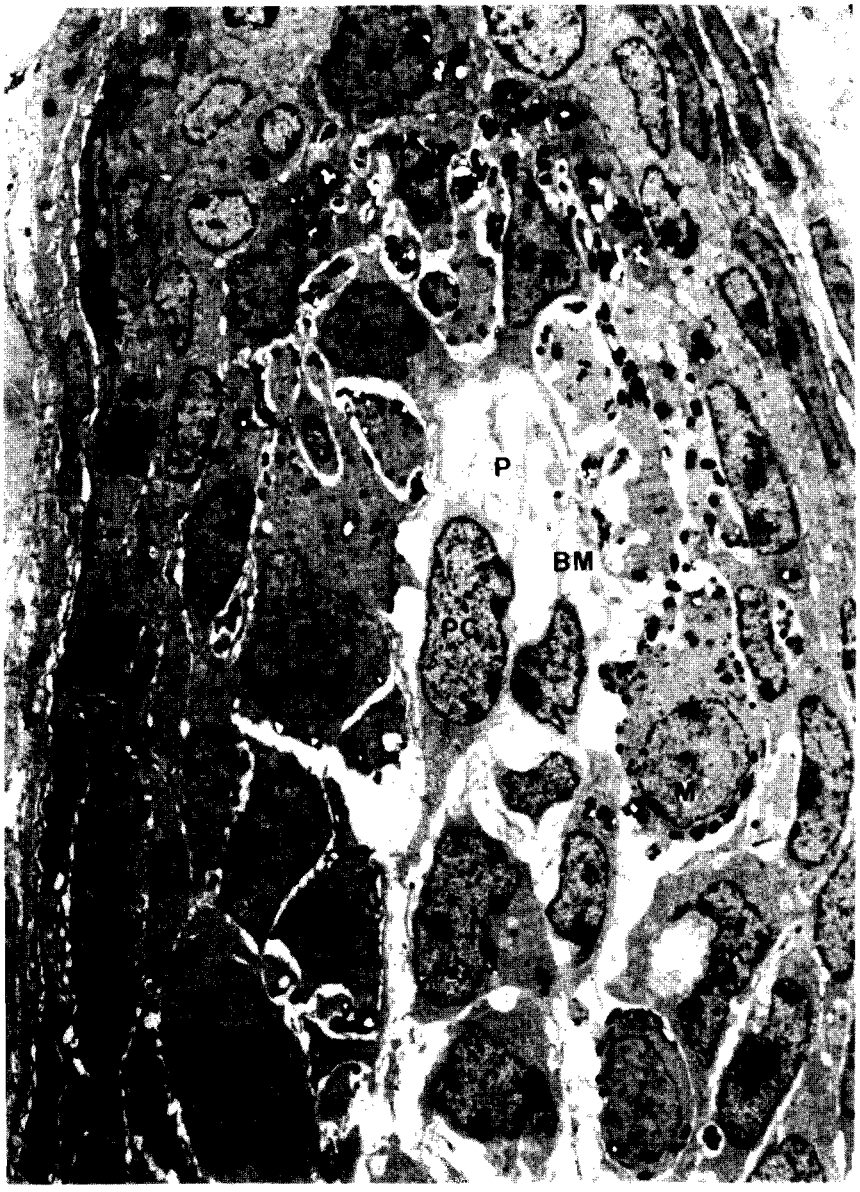

Fig. 4. Electron micrograph of the hair bulb of a 3-day-old C57BL/10J mouse. Hair bulb melanocytes (M) locate in the basal layers of the hair matrix in close proximity to the dermal papilla (P) and rest on the basement membrane (BM). A functional melanin unit between hair bulb melanocytes and keratinocytes (K) is formed. Numerous well-developed dendrites filled with stage IV melanosome are observed. KM: mitotic keratinocyte; PC: dermal papilla cell. x 4,000

phase of the hair (ca 17 days). This phase is divided into six substages (anagen I-IV: Chase, 1954). When cell proliferation of hair bulb keratinocytes ceases (catagen), the melanocytes also cease to produce melanosomes and no further cells enter the shaft as medulla. This stage is followed by the resting stage (telogen). In mice, hair growth is synchronized and proceeds in waves all over the body (Dry, 1926).

Hair bulb melanocytes differ from epidermal melanocytes by a larger size (Fig. 4), larger dendrites and a supplement of fewer keratinocytes in contrast to epidermal melanocytes (30-40 keratinocytes). In the mature hair follicle, a functional melanin unit between melanocytes and keratinocytes in the hair bulb is formed. Hair bulb melanocytes locate in the basal layers of the hair matrix in close proximity to the dermal papilla and rest on the basement membrane (Fig. 4). Melanogenesis is strictly coupled to the growth phase of the hair growth cycle (anagen III-VI, Chase, 1954). Melanogesis ceases early in catagen and is completely absent in telogen (Chase, 1954). Towards the end of anagen the number of identifiable melanocytes decreases and melanocytes lose their dendrites, shrink and become less pigmented, then they disappear in catagen (Chase, 1954). However, a small number of dedifferentiated cells (melanoblasts) positive to the combined dopa-premelanin reaction still remain in the hair bulbs (Hirobe, unpublished).

It has been reported that telogen follicles can be induced to enter new anagen by plucking hair shafts. The method induces the highly synchronized development of homogeneous anagen follicle populations over the entire depilated skin area. Silver et al. (1969) observed the depilation-induced anagen on epon-embedded, semi-thin sections of the skin of mice of strains C57BL/Ch, BUB/Wi, C3HP/Wi, DBA/1Ch and DS/Ch. A population of non-dividing «clear dendritic cells» of the hair germ immediately adjacent to the dermal papilla represented the melanoblasts of telogen hair follicles, which went on to produce melanin in anagen. These clear dendritic cells underwent mitosis before they resumed melanogenesis during anagen III-IV, and changed back into dormancy during telogen. Recently, molecular characteristics of melanogenesis during the depilation-induced hair growth cycle in mice have been studied. The levels of tyrosinase mRNA and protein as well as the melanosomal protein gp75 (mouse brown locus protein) were below detectability in telogen of C57BL/6 mice (Slominski et al., 1991). During the 1st and 2nd days of anagen melanin was absent, and gp75 and tyrosinase activity were also undetectable (Slominski et al., 1991). However, within 1 day of anagen induction, tyrosinase mRNA and protein were around the level of detectability, and were clearly identifiable by the 2nd day. Sugiyama (1979) also demonstrated by electron microscopic observations the presence of poorly differentiated melanocytes containing unmelanized, but dopa-positive premelanosomes in early anagen. The abundance of tyrosinase mRNA, protein and activity as well as gp75 increased rapidly by the 5th day, reaching a plateau by the 8th-12th days (Slominski et al., 1991). Burnett et al. (1969) also reported the presence of three isoforms of tyrosinase (T_1, T_2 and T_3) in anagen IV-VI. Melanin was first detected by the 4th-5th days of anagen and became abundant by the 8th-12th days (Slominsky et al., 1991). Burchill et al. (1989) also demonstrated that in C3H/He-A^{vy} (viable yellow) mouse skin, tyrosinase activity was dependent both on enzyme synthesis and post-translational modification during depilation-induced anagen. The activity of TRP-2 similarly increased at the early and middle anagen, and decreased at the late anagen of depilation-induced hair growth cycle of C57BL/6 mice (Slominski et al., 1994). However, the increase in TRP-2 activity was much smaller than that of tyrosinase activity, suggesting that tyrosinase is the major regulator of melanogenesis in hair growth cycle, whereas TRP-2 plays a modulatory role.

The mechanisms of the initiation of hair growth cycle are still unknown. Recently, Paus et al. (1994) reported that anagen-associated decline in the number of mast cells of C57BL/6 mice was correlated with the occurrence of substantial mast cell degranulation and the secretory products from the mast cell granules induced the development of new anagen follicles. These studies suggest that mast cells play an important role in the initiation of mouse hair growth cycle.

Regulation of the function of mouse melanocytes by hormone

Regulation of the function of epidermal melanocytes by hormone

Melanocyte-stimulating hormone (MSH), which is synthesized and secreted by the pars intermedia of the pituitary, has a stimulative effect of melanogenesis in the mouse skin (Pomerantz and Chuang, 1970; Geschwind et al., 1972; Levitin et al., 1979). In addition to the stimulative effects on mouse melanogenesis, MSH has been shown to induce the differentiation of melanocytes (Hirobe and Takeuchi, 1977a,b, 1978; Ito and Takeuchi, 1984). The number of melanocytes positive to the dopa reaction in the epidermis of newborn C57BL/10J mice was shown to increase after they were injected with α-MSH (Fig. 5, Hirobe and Takeuchi, 1977a). The increase in the number of dopa-positive cells in the epidermis after treatment with α-MSH seems to be the result of the induction of tyrosinase synthesis in unmelanized melanoblasts of the epidermis. The possibility that treatment with α-MSH leads to the proliferation of active melanocytes present in the epidermis at birth can be excluded by the observation that the number of dopa-positive cells after treatment with α-MSH was comparable to that of the melanoblast-melanocyte population in the epidermis (Hirobe and Takeuchi, 1977a). This increase in the number of melanocytes after treatment with α-MSH was completely suppressed

when mice were injected with actinomycin D or cycloheximide, suggesting that the hormone-induced differentiation requires de novo transcription and translation (Hirobe, 1983a).

The number of dopa-positive melanocytes also increased when skin explants from newborn mice were cultured with medium 199 supplemented with α-MSH (Hirobe and Takeuchi, 1977b). The number of dendrites and the total length of dendrites in epidermal melanocytes were also increased with skin explants were similarly cultured (Hirobe, 1978). On the basis of these studies, it is reasonable to conclude that α-MSH normally acts in newborn mice to stimulate the differentiation of melanoblasts into melanocytes by

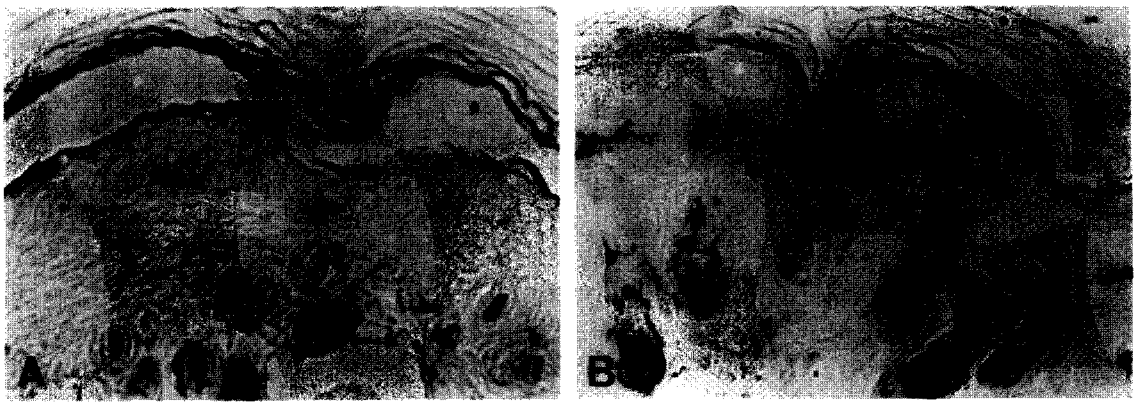

Fig. 5. Vertical sections of the dorsal skins of 3-day-old C57BL/10J mice. Dendritic cells positive to the dopa reaction are observed in the basal layer (short arrows) of epidermis and in the hair follicle (long arrows) of control **(A)** and α-MSH (1 μg/g BW)-treated **(B)** mice. Numerous dopa-positive cells with well-developed dendrites are seen 2 days after α-MSH injection. x 160

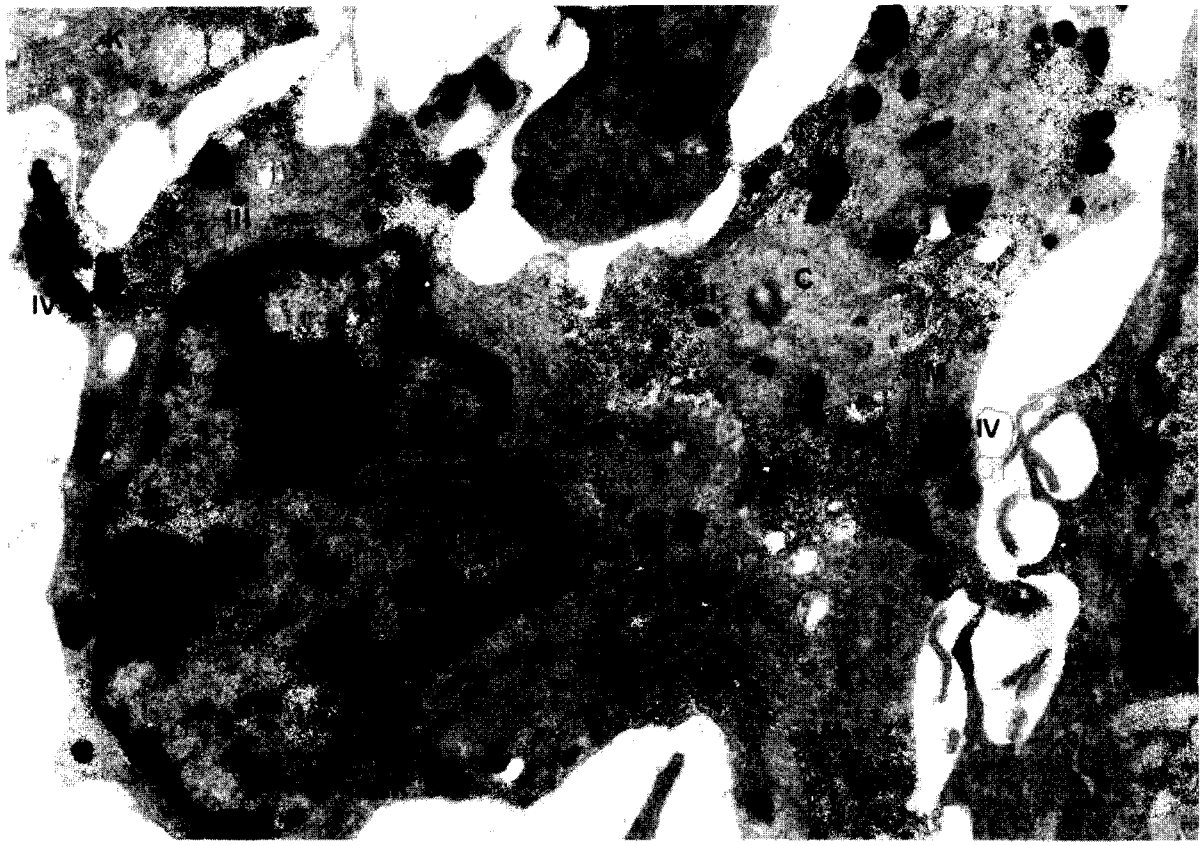

Fig. 6. Electron micrograph of an epidermal melanocyte (α-MSH-treated) of a 3-day-old C57BL/10J mouse. Numerous stage IV melanosomes (IV) are seen, particularly in the dendrite areas. III: stage III melanosome; C: centriole; G: Golgi apparatus; M: mitochondrion; RER: rough endoplasmic reticulum; K: basal keratinocyte. x 24,000

inducing tyrosinase activity, melanosome formation, transfer of melanosomes and increased dendritogenesis.

Dibutyryl adenosine 3',5'-cyclic monophosphate (DBcAMP) similarly induced the differentiation of melanocytes both in vivo (Hirobe and Takeuchi, 1977a) and in vitro (Hirobe and Takeuchi, 1977b). These results suggests that the action of MSH may be, in part, mediated through cAMP. This assumption is supported by the results with theophylline, which induced in organ culture an increase in the number of differentiated epidermal melanocytes, similar to that induced by α-MSH and DBcAMP (Hirobe and Takeuchi, 1977b).

The differentiation-stimulating effect of α-MSH was confirmed by observation using an electron microscope (Hirobe and Takeuchi, 1978). Newborn C57BL/10J mice were injected subcutaneously in the dorsum with α-MSH at 1 and 2 days after birth. The epidermis of α-MSH-treated 3-day-old mice contained melanocytes with numerous stage IV melanosomes and with no or only a few melanoblasts (Fig. 6, Hirobe and Takeuchi, 1978). Changes in other organelles of MSH-treated melanocytes were also noticeable. Golgi apparatus and RER decreased in amount during MSH-induced differentiation, though the number of mitochondria showed no notable change. The amount of SER increased significantly in MSH-treated cells (Hirobe and Takeuchi, 1978). These results suggest that Golgi apparatus and RER transform into other forms of organelles, including melanosomes and SER, during MSH-induced differentiation.

Regulation of the function of hair bulb melanocytes by hormone

Alpha-MSH is thought to play an important role in the regulation of melanogenesis of hair bulb melanocytes in anagen hair follicles in addition to epidermal melanocytes. It has been reported that injection of α-MSH to C3H/He-A^{vy} mice enhances the expression of tyrosinase mRNA transcripts (Burchill et al., 1993), tyrosinase synthesis (Burchill et al., 1988) and activity (Burchill and Thody, 1986) of hair follicular melanocytes at early and middle anagen of the first hair growth cycle of depilation-induced hair growth cycle. In addition, it has been documented that α-MSH and related analogue, [Nle^4-D-Phe^7]α-MSH (Sawer et al., 1980) induce a darkening of hair of C57BL/6J-A^y (lethal yellow) mice and it is accompanied with transformation of pheomelanogenesis to eumelanogenesis of hair bulb melanocytes (Geschwind et al., 1972; Tamate and Takeuchi, 1981; Levine et al., 1987; Imokawa et al., 1988; Granholm and Van Amerongen, 1991). Recently, it was reported that proopiomelanocortin (POMC) gene transcription, translation and product processing were dependent on the hair growth cycle of C57BL/6 mice (Slominski et al., 1992). POMC expression was observed only in anagen skin. Alpha-MSH is one of the products of POMC gene. Thus, the results suggest that α-MSH plays an important role in the regulation of melanogenesis of hair follicular melanocytes during hair growth cycle; namely it stimulates tyrosinase mRNA, protein and activity as well as melanin synthesis.

Response of mouse melanocytes to external stimuli

Response of melanocytes to skin wounding

It has been reported that the number of functioning melanocytes in the epidermis of mouse skin is increased by external stimuli, such as skin wounding (Rovee and Reams, 1964). However, whether the increase in the number of melanocytes is due to differentiation of precursor melanoblasts or to mitotic division of melanocytes was not resolved. In order to solve this problem, the effect of wounding the skin on mitosis in epidermal melanoblasts and melanocytes of C57BL/10JHir mice was investigated in detail (Hirobe, 1983b, 1988a,b). A full-thickness 7 mm-long cut was

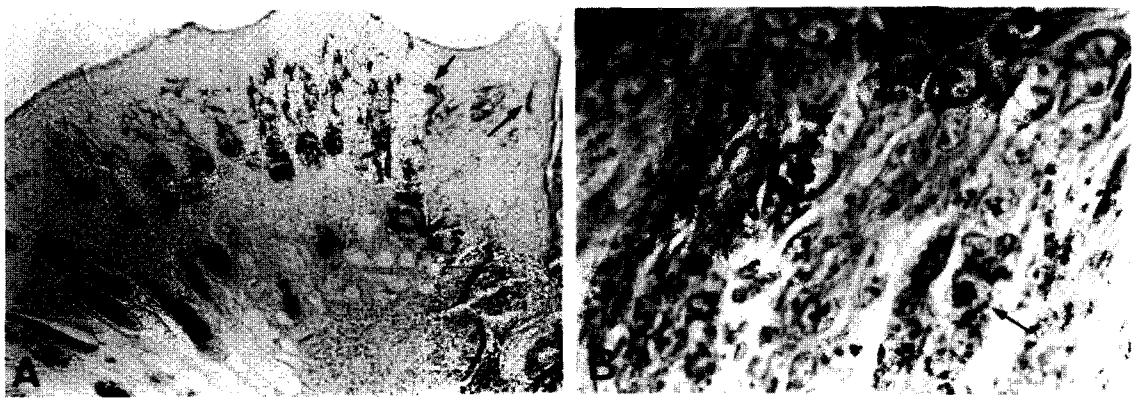

Fig. 7. A. Vertical section of the dorsal skin of a C57BL/10JHir mouse at 3 days after wounding. Numerous dopa-positive cells are seen in the vicinity of the wound (short arrow) and behind the advancing epidermal sheet (long arrow). Numerous dopa-positive cells with well-developed dendrites are seen in the epidermis and hair follicle. **B.** Vertical section of the dorsal skin of a C57BL/10JHir mouse at 3 days after wounding. Pigment-producing melanocytes in metaphase are observed in the epidermis (short arrow) and hair follicle (long arrow). Chromosomes are arranged equatorially and pigments localize peripherally. No dopa treatment. A, 120; B, x 460

made on the middorsal skin of 1.5-day-old mice using fine iridectomy scissors. In the epidermis within 1 mm from the wound edge, the melanocyte population positive to the dopa reaction (Fig. 7A) as well as the melanoblast-melanocyte population positive to the combined dopa-premelanin reaction increased dramatically up to 3 days, then gradually decreased (Hirobe, 1983b). In contrast, both populations in the regenerating wound epidermis appeared at 3 days and increased until the 7th day, then gradually decreased. However, the maximal population density in the regenerating epidermis did not exceed the initial density. The size of the melanocyte population in both epidermis did not differ from that of the melanoblast-melanocyte population in any stage of wound healing (Hirobe, 1983b). Moreover, pigment-producing melanocytes in mitosis (Fig. 7B) were found immediately after wounding in the epidermis within 1 mm from the wound edge, but not in the regenerating epidermis and control epidermis (Hirobe, 1983b). These results indicate that epidermal melanocytes in neonatal mouse skin can be stimulated to undergo mitosis immediately adjacent to a skin wound and, thereafter, to migrate into the regenerating epidermis.

It is not known whether the proliferative activity of differentiated melanocytes changes with development. To solve this problem the wounding of the skin was performed during the postnatal development of C57BL/10JHir mice. The results showed that the increase in the melanocyte and melanoblast-melanocyte populations as well as the increase in mitotic indices of pigment-producing melanocytes of older mice was reduced and delayed when compared to newborn mice (Hirobe, 1988a). These results suggest that the proliferative response of mouse epidermal melanocytes to skin wounding was diminished as developmental age advanced.

A question arises as to whether the proliferative response of mouse epidermal melanocytes during the healing of skin wounds is genetically determined. The number of epidermal melanocytes of newborn mice of strain C3H/HeJmsHir (agouti) was much fewer than that of C57BL/10JHir (Hirobe, 1982a). In order to get insights into the genetic control of melanocyte proliferation, the F_1, F_2 and backcross matings between C57BL/10JHir and C3H/HeJmsHir were performed. Effects of skin wounding were also studied in congenic C57BL/10JHir-A/A (agouti, Hirobe, 1986, 1991b). In the epidermis within 1 mm from the wound edge in C57BL/10JHir and C57BL/10JHir-A/A, the melanocyte population positive to the dopa reaction as well as the melanoblast-melanocyte population positive to the combined dopa-premelanin reaction increased dramatically until the 3rd day, then gradually decreased. In contrast, the melanocyte population of C3H/HeJmsHir did not increase after wounding, despite the fact that the melanoblast-melanocyte population slightly increased (Hirobe, 1988b). Pigment-producing melanocytes in mitosis were frequently found in C57BL/10JHir and C57BL/10JHir-A/A, but not in C3H/HeJmsHir. In the F_1 generation, the offspring from reciprocal crosses exhibited intermediate values in both populations at 3 days after wounding. The F_2 generation included the C3H/HeJmsHir type, F_1 type and C57BL/10JHir type in a ratio of 1:2:1 in both populations. Moreover, both reciprocal backcrosses gave 1:1 ratios of parent type to F_1 type in both populations (Hirobe, 1988b). These results indicate that the proliferative activity of mouse epidermal melanocytes during the healing of skin wounds is controlled by semidominant genes.

It is also reported that semidominant genes are involved in regulating the melanocyte and melanoblast-melanocyte populations in the epidermis of newborn mouse skin by genetical study using C57BL/10JHir and C3H/HeJmsHir mice (Hirobe, 1982a). It seems likely that the genes controlling the melanocyte and melanoblast-melanocyte populations in the epidermis of newborn mouse skin are identical to the genes controlling the proliferative activity of epidermal melanocytes during the healing of skin wounds, since the similar histogram pattern of melanocyte and melanoblast-melanocyte populations between the newborn mouse skin (Hirobe, 1982a) and the skin during wound healing (Hirobe, 1988b) was observed in the offspring of F_1, F_2 and backcross generations. The genes are thought to determine the proliferative activity of epidermal melanocytes.

It is not known at present what substances are involved in initiating mouse melanocyte proliferation after skin wounding. Basic fibroblast growth factor (bFGF) is one of the key signal molecules for transforming the mechanical injury into a chemical one that can initiate cell proliferation (it may stimulate the semidominant genes), since bFGF is released from a cytoplasmic storage site into the extracellular environment via diffusion after wounding (Clarke et al., 1993).

Response of melanocytes to ultraviolet radiations

Ultraviolet (UV) radiation is one of the important factors that stimulate melanogenesis in mice. Earlier observations have shown that the production and transfer of melanin pigment is enhanced by UV irradiation. In the hairless pigmented adult mice of strain C57HR/Ch, UV irradiation led to the development of an intense hyperpigmentation both in the dorsal skin and in the dorsal surface of the tail and feet (Quevedo and Smith, 1963). The number of melanocytes increased in the irradiated skin. In the plantar skin of UV-irradiated hairy mice of strains L/St, CBA/St, C57BL/St, C3H/St, PBR/St, BRS/St, IPBR/St and DBL, the number of epidermal melanocytes increased dramatically (Quevedo and Smith, 1963). Long-wave UV (UVA, 320-400 nm) differs from short-wave UV (UVB, 290-320 nm) in the time course of skin hyperpigmentation after irradiation. Visible pigmentation resulting from UVA irradiation is immediate, while the pigmentation after UVB irradiation

is delayed for several days. Following UV irradiation the number of epidermal melanocytes in the epidermis of ears (UV, Rosdahl and Szabo, 1978) and tail (UVB, Blog and Szabo, 1979b) of hairy mice of strain C57BL/6J also increased. The increase in the number of epidermal melanocytes in the tail skin was observed in C57BL/6J mice after exposure to psolaren followed by UVA irradiation (Blog and Szabo, 1979a).

UV causes both an increase in melanogenesis and an increase in the number of differentiated melanocytes in mice. The increase in the number of melanocytes may be the result of the induction of melanocyte differentiation in pre-existing melanoblasts and/or the stimulation of the proliferation of melanocytes (Sato and Kawada, 1972; Rosdahl, 1978).

The mechanism of UV-induced melanogenesis in mice is still unknown. However, recent studies have implied that MSH-receptor mechanism is involved in regulating the UV-induced pathway. UV and topically applied MSH acted synergistically to increase skin darkening and melanin content in the skin of hairless mice of strain BOM (Bolognia et al., 1989). Dopa-histochemistry showed that UVB acted synergistically with MSH to increase the number of differentiating melanocytes in the epidermis. These results suggest the possibility that the effects of UV radiation are mediated through increased MSH receptor activity. The relationship between UV radiation and MSH receptors in the stimulation of pigmentation need not be a direct one and could involve many other biochemical systems within the skin. Serum-free culture methods for melanoblasts and melanocytes of hairless mice of F_1 (HR/De female x HR/1 male) will be a useful tool for the study of the mechanism of action of UV (Furuya et al., 1993).

It is not known whether UV irradiation stimulates the melanogenesis of hair bulb melanocytes in the mouse skin. It remains to be investigated in a future study.

Response of melanocytes to ionizing radiations

Ionizing radiations are thought to be one of the important environmental factors that affect the structure and function of mouse melanocytes. They have been reported to exert a dual action on differentiated mouse melanocytes: selective killing (Chase, 1949; Potten, 1968; Reams and Schaeffer, 1968); and stimulation of melanogenesis (Quevedo and Grahn, 1958; Quevedo and Isherwood, 1958). Recently, the effects of γ-radiations on embryonic mouse melanoblasts have been studied (Hirobe and Zhou, 1990; Hirobe, 1994c). Gamma-irradiation brought about an abnormal structure of hair follicular melanocytes of 3.5-day-old F_1 (C57BL/10JHir-p/p female x C57BL/10JHir male) mice (fully pigmented but abnormally round in morphology, Fig. 8A,B), and that the greatest effect was observed at 6.5 days of gestation. Abnormal round melanocytes were found both in the hair matrix (Fig. 8A) and the dermal papilla (Fig. 8B, Hirobe and Zhou, 1990). The frequency of the abnormal hair follicular melanocytes increased in a dose-dependent manner (Hirobe and Zhou, 1990).These results suggest that γ-radiation affects dendritogenesis and the location of mouse melanocytes in the hair follicles, with greater effects seen at the earlier stages of development. On the other hand, γ-irradiation brought about an abnormal white spot (Fig. 8C) in mid-ventrum of 25-day-old F_1 (C57BL/10JHir-

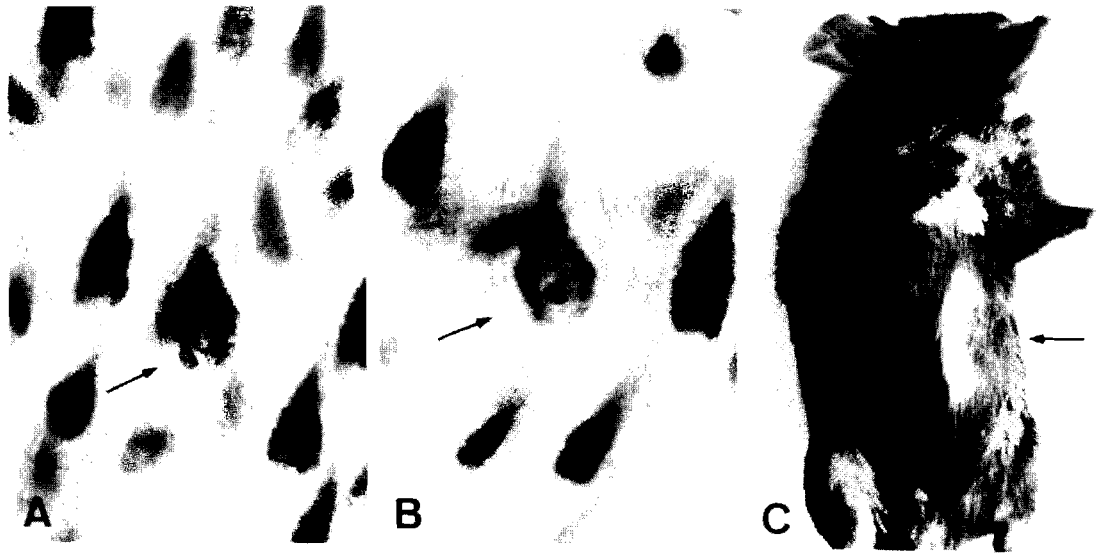

Fig. 8. A, B. Whole mount preparations of the dorsal skin of 3.5-day-old mice showing hair follicles. Round melanocytes (arrows) are observed in the hair matrix (A) and the dermal papilla (B) in hair follicles of the dorsal skin of F_1 offspring (C57BL/10JHir-p/p female x C57BL/10JHir male). Pregnant females were irradiated with a single dose of γ-radiation (1.00 Gy) at 10.5 days of gestation. Other follicles are normal. x 210. **C.** A white spot (arrow) is seen in the mid-ventrum of 25-day-old F_1 offspring (C57BL/10JHir-p/p female x C57BL/10JHir male). Pregnant females were irradiated with a single dose of γ-radiation (1.25 Gy) at 8.5 days of gestation.

p/p female x C57BL/10JHir male) (Hirobe, 1994c). Melanoblasts and melanocytes were not observed in the spotted skin. The frequency of the spots increased in a dose-dependent manner. White spots were also found in mid-ventrum of 25-day-old F_1 (C57BL/6J female x C3H/HeJmsHir male) exposed to γ-rays (Hirobe, 1994c). However, the frequency of the spots in F_1 (C57BL/6J female x C3H/HeJmsHir male) was extremely lower than that in F_1 (C57BL/10JHir-p/p female x C57BL/10JHir male), suggesting the possibility that the frequency of mid-ventral white spots is genetically controlled. Moreover, the highest frequency was found in F_1 (C57BL/10JHir-p/p female x C57BL/10JHir male) irradiated at 8.5 days of gestation (Hirobe, 1994c). This stage corresponds to the stage of initiation of neural-crest cell migration (Rawles, 1947). These results indicate that γ-radiation affects the differentiation of melanocytes in the skin both with genetic control and with greater effects seen at the stage of initiation of neural-crest cell migration. Similar differences in the yield of mid-ventral spots in mice of different genetic backgrounds exposed prenatally to X-rays were reported (Russell and Major, 1957; Fahrig, 1975). The results of Hirobe and Zhou (1990) suggest that the most sensitive time for the induction of abnormal hair follicular melanocytes corresponds to the developmental stage before the initiation of neural tube formation. The time sensitive to the induction of mid-ventral white spots seems to be different from that sensitive to the induction of abnormal differentiation of melanocytes.

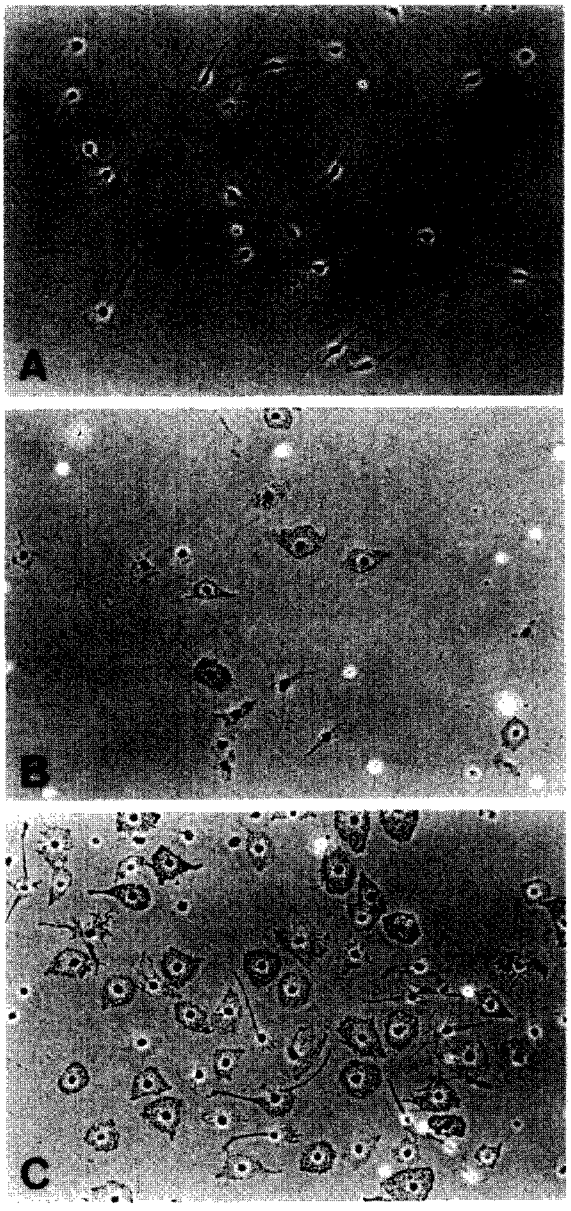

Fig. 9. Primary cultures of epidermal cell suspensions derived from the dorsal skin of C57BL/10JHir mice in three different media: control (MDM alone, **A**), 10 nM α-MSH **(B)** and 0.5 mM DBcAMP **(C).** After 12 days in culture enriched cultures of pure melanoblasts (A) or melanocytes (B, C) are obtained. They are dendritic, polygonal or epithelioid in morphology. Almost all keratinocytes that existed at early stage of primary culture died around 12 days. Phase contrast microscopy. x 120

Serum-free culture of mouse epidermal melanoblasts and melanocytes

Serum-free culture of melanoblasts

In order to understand the mechanism involved in regulating the melanocyte differentiation, the development of a serum-free culture system to optimize and maintain melanoblasts from epidermal cell suspensions and the survey of the effects of stimulating factors on them are particularly useful. Disaggregated epidermal cell suspensions from dorsal skin of 0.5-day-old C57BL/10JHir mice were cultured in melanoblast-defined medium (MDM) consisting of Ham's F-10 medium supplemented with insulin (Ins), bovine serum albumin (BSA), ethanolamine (EA), phosphoethanolamine (PEA) and sodium selenite (SE). MDM permitted the selective proliferation of melanoblasts from dissociated epidermal cells. MDM maintained these melanoblasts in a slowly proliferated and undifferentiated state for at least 2 weeks (Hirobe, 1992c). The keratinocyte colony that existed in the early stage decreased gradually and disappeared completely around 12 days (Fig. 9A). These results suggest that Ins, BSA, EA, PEA and SE are essential for maintaining melanoblasts in vitro.

Induction of melanocyte differentiation

Alpha-MSH supplemented into MDM from the initiation of primary culture induced the differentiation of melanoblasts into melanocytes (Hirobe, 1992c). Differentiated melanocytes appeared around 3-4 days and almost all cells differentiated around 7-8 days of culture. After 12 days pure cultures of melanocytes were obtained (Fig. 9B), but no stimulation of melanocyte

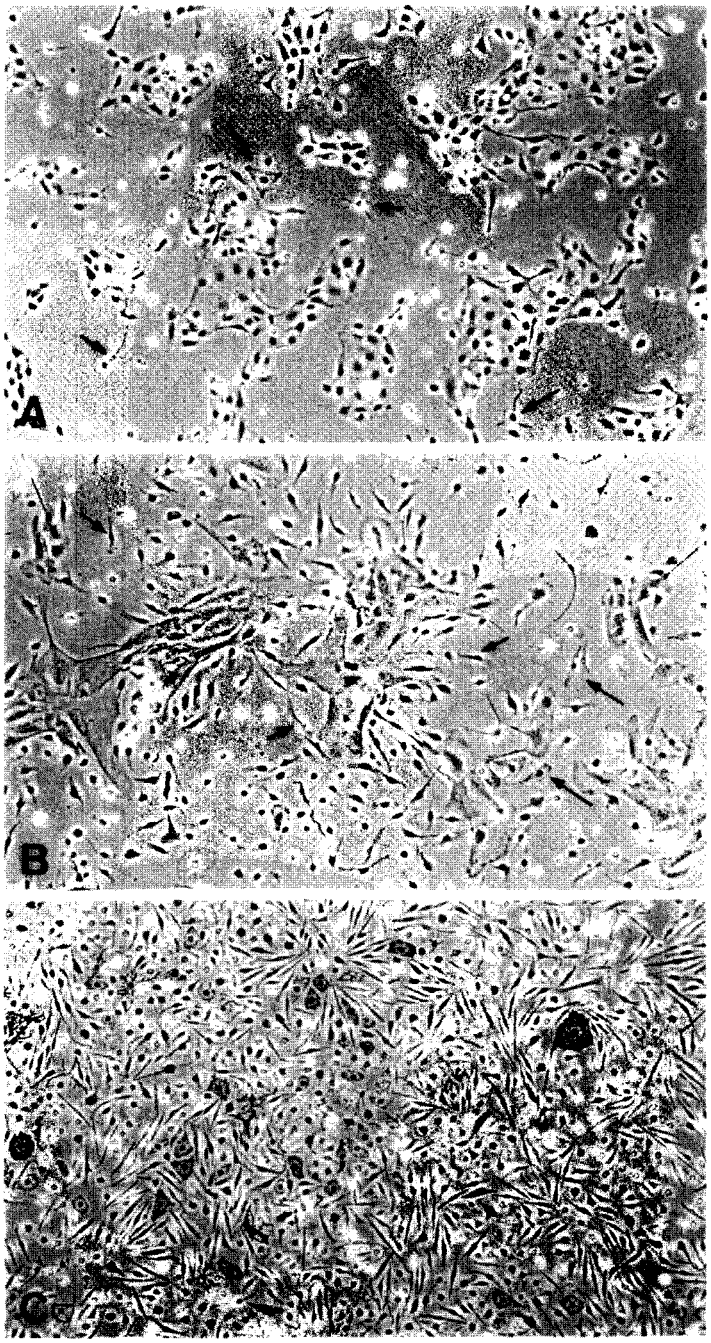

Fig. 10. Primary cultures of epidermal cell suspensions derived from the dorsal skin of C57BL/10JHir mice in MPM. **A.** After 2 days in culture. Keratinocyte colonies and a small number of melanoblasts (short arrows) and melanocytes (long arrows) are evident. The melanoblasts are bipolar or tripolar. **B.** After 6 days in culture. Melanoblasts (short arrows) have increased in number, and a small number of melanocytes (long arrows) are seen. Keratinocyte colonies increased in size and number. Mitotic melanoblasts (arrowhead) are often observed. **C.** After 12 days in culture. Enriched culture of pure melanoblasts and melanocytes. No keratinocytes are observed. Phase contrast microscopy. x 100

proliferation was observed. DBcAMP (Fig. 9C) supplemented into MDM also induced the differentiation of melanocytes (Hirobe, 1992c). The action of MSH may be mediated through cAMP, since isobutylmethylxanthine (IBMX), a potent inhibitor of cAMP phosphodiesterase, similarly induced the differentiation of melanocytes, and the inhibitor acted synergistically with α-MSH or DBcAMP to induce melanocyte differentiation (Hirobe, 1992c).

Induction of the proliferation of meanoblasts and melanocytes

DBcAMP supplemented into MDM at high doses induced the proliferation of melanocytes in primary culture (Hirobe, 1992c). Numerous mitotic melanocytes were observed in the dishes cultured with DBcAMP. After 12 days pure populations of differentiated melanocytes could be harvested (Fig. 9C). A more than 3- to 4-fold increase in the number of melanocytes was observed from 1 to 12 days. On the other hand, when the epidermal cell suspensions were cultured with MDM supplemented with DBcAMP and bFGF (melanoblast proliferation medium, MPM), melanoblasts proliferated dramatically around keratinocyte colonies (Fig. 10A,B) and after 12 days, pure and enriched cultures of melanoblasts (ca. 75%) and melanocytes (ca. 25%) were obtained (Fig. 10C). Numerous mitotic melanoblasts were observed (Fig. 10B). A more than 30-fold increase in the number of melanoblasts and melanocytes was observed from 1 to 12 days (Hirobe, 1992a). These results suggest that bFGF is essential for the sustained proliferation of mouse epidermal melanoblasts in culture. The possibility exists that the proliferation of mouse epidermal melanocytes during the healing of skin wounds (Hirobe, 1983b, 1988a,b) is regulated by bFGF and the semidominant genes (Hirobe, 1988b) involved in regulating the proliferation of mouse epidermal melanocytes are stimulated by bFGF. However, this hypothesis remains to be investigated in a future study.

Regulation of melanocyte differentiation by keratinocytes

In order to clarify the role of keratinocyte in melanocyte differentiation, pure cultures of melanoblasts were co-cultured with keratinocytes. Pure and enriched cultures of primary keratinocytes were obtained by culturing epidermal cell suspensions of C57BL/10JHir with keratinocyte-defined medium (KDM) consisting of Ca^{2+}-free minimum essential medium (MEM) supplemented with MEM-nonessential amino acid solution, Ins, BSA, EA, PEA, SE, epidermal growth factor, hydrocortisone, dexamethasone and 0.03 mM $CaCl_2$ (Hirobe, 1992c). The primary keratinocytes were trypsinized and seeded into the pure melanoblasts obtained in MDM at 12 days (Hirobe, 1992c). The results showed that α-MSH or IBMX induced the differentiation of melanoblasts into melanocytes in the presence of keratinocytes but not in the absence of

keratinocytes. These results support the earlier observations that α-MSH induces the differentiation of mouse epidermal melanocytes in vivo (Hirobe and Takeuchi, 1977a, 1978) and in skin organ culture (Hirobe and Takeuchi, 1977b) and, in addition, add a new finding that MSH induces the differentiation of mouse epidermal melanocytes in the presence of keratinocytes. Induction of melanocyte differentiation by MSH does not necessarily require the presence of dermal components.

On the other hand, treatment with DBcAMP at high doses, as well as combined treatment of MSH and IBMX, induced the differentiation of melanocytes in the absence of keratinocytes (Hirobe, 1992c). These results show the possibility that increased cAMP induces melanocyte differentiation. MSH alone or IBMX alone may not be able to maintain the concentration of cAMP high enough to induce melanocyte differentiation. It is possible that keratinocyte-derived factors may potentiate MSH by inhibiting cAMP phosphodiesterase, similar to IBMX.

Regulation of the proliferation of melanoblasts and melanocytes by keratinocytes

The primary keratinocytes were similarly trypsinized and seeded into the pure melanoblasts obtained in MDM at 12 days. DBcAMP supplemented into MDM at high doses induced the proliferation of melanocytes in the presence of keratinocytes, but not in the absence of keratinocytes (Hirobe, 1992c). These results suggest that keratinocytes produce factors which stimulate the proliferation of mouse epidermal melanocytes in an increased cAMP level.

Pure and enriched populations of melanoblasts and melanocytes cultured in MPM were trypsinized and seeded into new dishes. At 1 day of secondary culture of pure melanoblasts and melanocytes in MPM, subconfluent primary keratinocytes in KDM were trypsinized and seeded. The melanoblasts and melanocytes proliferated well in the presence of keratinocytes but not in the absence of keratinocytes (Hirobe, 1992a). Moreover, the ability of keratinocytes to attach to substrate which greatly influenced successful co-culture of melanoblasts and keratinocytes was developmentally regulated (Hirobe, 1994a). When a substrate (e.g. type I collagen) which allowed keratinocyte attachment was used, then keratinocytes from older mice also attached well and melanocytes proliferated dramatically around the keratinocyte colonies (Hirobe, 1994a). The observation that melanoblasts proliferated well in the presence of keratinocytes suggests that keratinocytes produce factors that induce the proliferation of mouse epidermal melanoblasts. Basic FGF did not replace the proliferation-stimulating effect of keratinocytes (Hirobe, 1991a, 1992a). Mouse epidermal melanoblasts proliferated in the areas around the keratinocyte colonies both in the primary and secondary culture (Hirobe, 1992a). Moreover, conditioned medium prepared from pure and enriched cultures of keratinocytes failed to stimulate proliferation of melanoblasts in MPM (Hirobe, 1992a). These results suggest that the stimulation of proliferation of mouse epidermal melanoblasts by keratinocytes requires a direct contact between melanoblasts and keratinocytes. Therefore, it is reasonable to assume that the mitogenic factor derived from keratinocytes is not a paracrine factor but a membrane-bound factor.

In order to get some information about the keratinocyte-derived factors, numerous substances were added to MPM at 12 days of primary culture and tested for their mitogenic activity. Activators and inhibitors of protein kinase C, which is important for the regulation of cell proliferation (Nishizuka, 1988), were shown to replace the proliferation-stimulating effects of keratinocytes (Hirobe, 1994b). In contrast, no increase in the number of melanoblasts was observed in the dishes cultured with MPM supplemented with both the PKC activator and the inhibitor. These results suggest that the proliferation of mouse epidermal melanoblasts in culture is regulated by activating or inhibiting the activity of PKC. It is assumed that the keratinocyte-derived factors are involved in regulating the proliferation of melanoblasts by activating or inhibiting the PKC activity. However, this hypothesis remains to be investigated in a future study.

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