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Changes of Melanosome Morphology Associated With the Differentiation of Epidermal Melanocytes in Slaty Mice

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ABSTRACT

The slaty (Dct^{slt}) mutation is known to reduce the activity of dopachrome tautomerase, which converts dopachrome to 5,6-dihydroxyindole-2-carboxylic acid in the pathway of eumelanin synthesis and to inhibit melanosome maturation in melanocytes. However, it is not known whether the inhibition of melanosome maturation in slaty melanocytes is developmentally regulated. To address this point, changes in the morphology and maturation of melanosomes in cultured epidermal melanocytes derived from newborn mice of wild-type (black) and slaty mutant were surveyed under the electron microscope. In black melanocytes (Dct^{+}), almost all melanosomes were elliptical stage IV melanosomes. However, in slaty melanocytes, numerous spherical stage III melanosomes with globular depositions of pigment in addition to elliptical stage III melanosomes with intraluminal fibrils were observed. Mixed-type melanosomes containing both globular deposition and intraluminal fibrils of pigment were also observed. In slaty melanocytes, spherical and mixed-type melanosomes were gradually decreased after birth, whereas elliptical melanosomes were gradually increased. Stage IV melanosomes were very few in slaty melanocytes, and the number did not increase after birth. These results suggest that the slaty mutation blocks the melanosome maturation at stage III and affects the melanosome morphology (elliptical or spherical) in a developmental stage-specific manner. Anat Rec, 290:981–993, 2007. © 2007 Wiley-Liss, Inc.

Key words: slaty; melanocyte; culture; melanosome; epidermis; Golgi apparatus; mitochondria; lysosome

The mouse slaty (Dct^{Slt} or Slt) mutation is a recessive autosomal mutation (chromosome 14, approximately 5 cM from piebald [s]; Silvers, 1979). This mutation occurred in a heterogeneous stock carrying limb-deformity (ld^{J}) and mahogany (mg; Silvers, 1979). On a nonagouti background, slaty homozygotes possess a slightly diluted coat and slightly yellowish ears (Green, 1972). In addition to the original slaty mutation, two other mutations, slaty light ($Dct^{Slt\text{-}lt}$ or Slt^{lt}) and slaty 2J ($Dct^{Slt\text{-}2J}$ or Slt^{2J}) have been identified (Budd and Jackson, 1995). The $Dct^{Slt\text{-}lt}$ mutation possesses a more severe effect on coat color and is semidominant, and the mouse mutant for $Dct^{Slt\text{-}2J}$ is similar in phenotype to the slaty mutant mouse (Budd and Jackson, 1995). The slaty mutation is known to change an arginine to a glutamine in the first copper binding domain of dopachrome tautomerase (DCT), which converts dopachrome (DC) to 5,6-dihydroxyindole-2-carboxylic acid

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(DHICA) in the pathway of eumelanin synthesis (Korner and Pawelek, 1980; Jackson et al., 1992; Tsukamoto et al., 1992) and to yield about 10 to 30% activity of wild-type DCT in eye extracts (Jackson et al., 1992). DCT was originally identified as tyrosinase (TYR)-related protein-2 (TRP-2) that maps to the mouse slaty locus (Jackson et al., 1992; Tsukamoto et al., 1992). DCT is produced from both wild-type and slaty mutant cDNA, but the protein level of DCT in the slaty mutant is extremely lowered (Kroumpouzos et al., 1994). The reduced eumelanin synthesis in the slaty mutant was confirmed by recent study using murine slaty epidermal melanocytes in serum-free primary culture (Hirobe et al., 2006). Costin et al. (2005) reported that immortalized slaty epidermal melanocytes possessed stage II and III eumelanosome-like melanosomes. However, detailed melanosome biogenesis during the postnatal development of slaty melanocytes is not well studied. These circumstances prompted us to investigate in detail the changes in the distribution, characteristics, and morphology of melanosomes and other organelles in cultured epidermal melanocytes derived from newborn mice of wild-type (black) and slaty mutant mice by using electron microscopy.

MATERIALS AND METHODS

Mice

All animals used in this study belonged to strain C57BL/10JHir-Dct^{+}/Dct^{+} (wild-type, black) and its congenic, C57BL/10JHir-Dct^{Slt}/Dct^{Slt} (mutant, slaty) of the house mouse, *Mus musculus*. C57BL/6J-Dct^{Slt}/Dct^{Slt} mouse (kindly supplied by Dr. M.L. Lamoreux, Texas A & M University, College Station, TX) was crossed with C57BL/10JHir-Dct^{+}/Dct^{+}, and congenic C57BL/10JHir-Dct^{Slt}/Dct^{Slt} mouse strain has been established by continued backcrossing for nine times followed by sib mating. The genic constitution of the line differs only in the slaty locus (Hirobe, 2003; unpublished results). The mice were given water and a commercial diet, OA-2 (Clea Japan, Tokyo, Japan) ad libitum. They were maintained at 24 ± 1°C with 40–60% relative humidity; 12 hr of fluorescent light was provided daily. The present study was approved by the ethics committee of the National Institute of Radiological Sciences in accordance with the guidelines of the National Institute of Health.

Melanocyte Primary Culture

The sources of tissue for the culture of melanocytes were dorsal skins from 0.5-, 3.5-, and 7.5-day-old black and slaty mice. Unless stated otherwise, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO). The method for obtaining epidermal cell suspensions was reported previously (Hirobe, 1992). Disaggregated epidermal cell suspensions were pelleted by centrifugation and suspended in Ham's F-10 medium (Gibco, Grand Island, NY). The cell pellets after centrifugation were resuspended in a melanocyte-proliferation medium (MDMD) consisting of Ham's F-10 medium supplemented with 10 μg/ml insulin (bovine), 0.5 mg/ml bovine serum albumin (Fraction V), 1 μM ethanolamine, 1 μM phosphoethanolamine, 10 nM sodium selenite, 0.5 mM dibutyryl adenosine 3′:5′-cyclic monophosphate (DBcAMP), 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 50 μg/ml gentamycin sulfate, and 0.25 μg/ml amphotericin B. The same lots of these supplements were used in this study. The cells in the epidermal cell suspension were counted in a hemocytometer chamber and plated onto dishes coated with type I collagen (Becton Dickinson, Bedford, MA) at an initial density of 1×10^6 cells/35-mm dish (1.04×10^5 cells/cm^2). Cultures were incubated at 37°C in a humidified atmosphere composed of 5% CO_2 and 95% air (pH 7.2). Medium was replaced by fresh medium four times a week. After 14 days, almost pure cultures of melanocytes were obtained.

Electron Microscopy

Primary melanocytes in pure culture for 14 days in MDMD were treated with a solution of 0.05% trypsin (Difco, Sparks, MD) and 0.02% ethylenediaminetetraacetate (EDTA) in Ca^{2+}-, Mg^{2+}-free phosphate buffered saline (CMF-PBS) at 37°C for 10 min. After trypsinization was inhibited by addition of 2,000 U/ml of soybean trypsin inhibitor, the cell suspensions were centrifuged at 1,500 rpm for 5 min (Hirobe and Abe, 2000). The cell pellets were fixed in chilled (2°C) 2.5% glutaraldehyde (Wako, Osaka, Japan) solution in 0.1 M phosphate buffer, pH 7.4. After washing with chilled 0.1 M phosphate buffer, cells were postfixed in chilled 1% osmium tetroxide (Taab Laboratories Equipment Ltd., Berkshire, UK) in 0.1 M phosphate buffer. After washing with chilled 0.1 M phosphate buffer, the cells were dehydrated in a series of graded ethanols and embedded in epoxy resin (Taab Laboratories Equipment Ltd.). Ultrathin sections were cut with a diamond knife on an ultramicrotome (Leica, Heerbrugg, Switzerland), stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (H-7600, Hitachi, Tokyo, Japan). This method was shown not to affect the morphology of melanosomes, the Golgi apparatus, and mitochondria (Hirobe and Abe, 1999, 2000; Hirobe et al., 2002). A total of 35 (0.5 days), 41 (3.5 days), and 39 (7.5 days) black melanocytes as well as 44 (0.5 days), 36 (3.5 days), and 56 (7.5 days) slaty melanocytes were surveyed for the presence of stage I, II, III, and IV melanosomes as well as the Golgi apparatus, mitochondria, and lysosomes. In the electron micrograph, the areas were measured and the numbers of melanosomes and other organelles were calculated per unit area (100 μm^2). Total numbers of melanosomes counted were 2,377 (0.5 days), 2,011 (3.5 days), and 1,962 (7.5 days) for black melanocytes and 1,879 (0.5 days), 2,889 (3.5 days), and 2,951 (7.5 days) for mutant melanocytes. Because the area of slaty melanocytes in cross-sections did not differ from that of black melanocytes and the area of melanocytes derived from 0.5-, 3.5-, and 7.5-day-old mice in cross-sections did not differ each other, the numbers of melanosomes and other organelles counted reflect the number of organelles present in unit area, rather than the density of organelles per unit area. The definitions of spherical and elliptical stages I, II, III, and IV melanosomes as well as of mixed melanosomes are as follows. Spherical stage I melanosomes possess vesicles within them, whereas elliptical stage I melanosomes possess intraluminal fibrils. The stage I melanosomes can be distinguished from lysosomes by their morphology and size. Lysosomes are larger than stage I melanosomes and possess amorphous internal structures. Spherical stage II

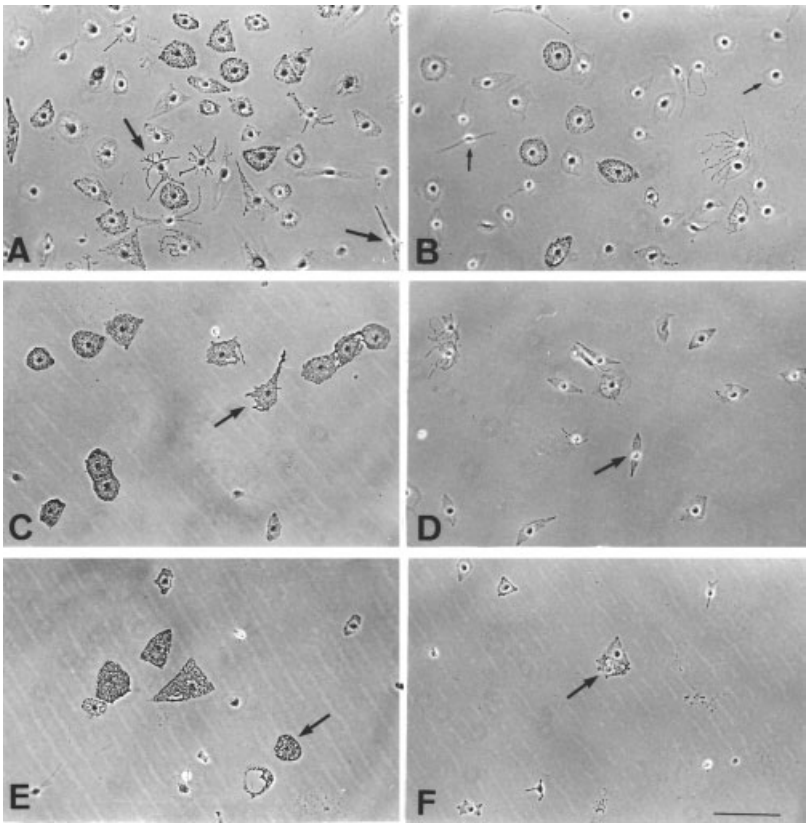

Fig. 1. **A–F:** Melanoblasts and melanocytes cultured in melanocyte-proliferation medium (MDMD) for 14 days derived from epidermal cell suspensions of 0.5- (A,B), 3.5- (C,D), and 7.5- (E,F) day-old black (A,C,E) and slaty (B,D,F) mice. Undifferentiated melanoblasts (small arrows) are bipolar, tripolar, or epithelioid and observed in 0.5-day-old slaty (B) cells, whereas the cells derived from 3.5- (C,D) and 7.5- (E,F) day-old mice are mostly differentiated melanocytes both in black and slaty mice. Differentiated melanocytes (large arrows) are dendritic, polygonal, or epithelioid in morphology. The number of melanoblasts and melanocytes is decreased as age advances. Phase-contrast microscopy. Scale bar = 100 μm.

melanosomes possess many vesicles within them, whereas elliptical stage II melanosomes possess completed intraluminal fibrils. Spherical stage III melanosomes possess globular depositions of pigment, whereas elliptical stage III melanosomes possess longitudinal depositions of pigment. The rims of fully pigmented spherical stage IV melanosomes are rough, whereas the rims of fully pigmented elliptical stage IV melanosomes are smooth. Mixed melanosomes possess both granular depositions of pigment and longitudinal depositions of pigment within one unit membrane. The statistical significance of the differences in the number of melanosomes and other organelles and in percentages of melanosomes was determined by Student's *t*-test for comparisons of groups of unequal size.

RESULTS

Light Microscopic Observations

Within 1–2 days after the initiation of primary culture of epidermal cell suspensions derived from dorsal skin of 0.5-day-old black mice in MDMD, undifferentiated melanoblasts without pigments that are bipolar, tripolar, or dendritic in morphology were in contact with adjacent keratinocyte colonies by means of a dendrite process. After 3 days, the keratinocyte colonies increased in size, and pigment-producing differentiated melanocytes appeared around the keratinocyte colonies. Melanocytes rapidly increased in number, and almost all cells differentiated around 7–9 days. After 14 days, almost all keratinocytes died and pure cultures of differentiated melanocytes were obtained (Fig. 1A). In slaty mice, a similar tendency of keratinocyte proliferation as well as melanocyte proliferation was observed. There were no differences in the number and shape of melanocytes (Fig. 1B) compared with wild-type melanocytes. However, in cultures derived from slaty mice (Fig. 1B), numerous cells with an undifferentiated phenotype were apparent, suggesting that the differentiation of melanocytes may be inhibited in a significant proportion of cells.

When the epidermal cell suspensions derived from 3.5- (Fig. 1C) and 7.5- (Fig. 1E) day-old black mice were cultured in MDMD, a similar tendency of keratinocyte proliferation as well as melanoblasts or melanocyte proliferation was observed. After 14 days, almost all keratinocytes died and pure cultures of differentiated melanocytes were obtained (Fig. 1C,E). The pigmentation increased compared with melanocytes from 0.5-day-old mice. In contrast, morphology of melanoblasts and melanocytes derived from 3.5- and 7.5-day-old slaty mice was similar to that derived from 0.5-day-old slaty mice, although the percentage of differentiated melanocytes in the melanoblast–melanocyte population gradually increased as age advanced (Fig. 1D,F).

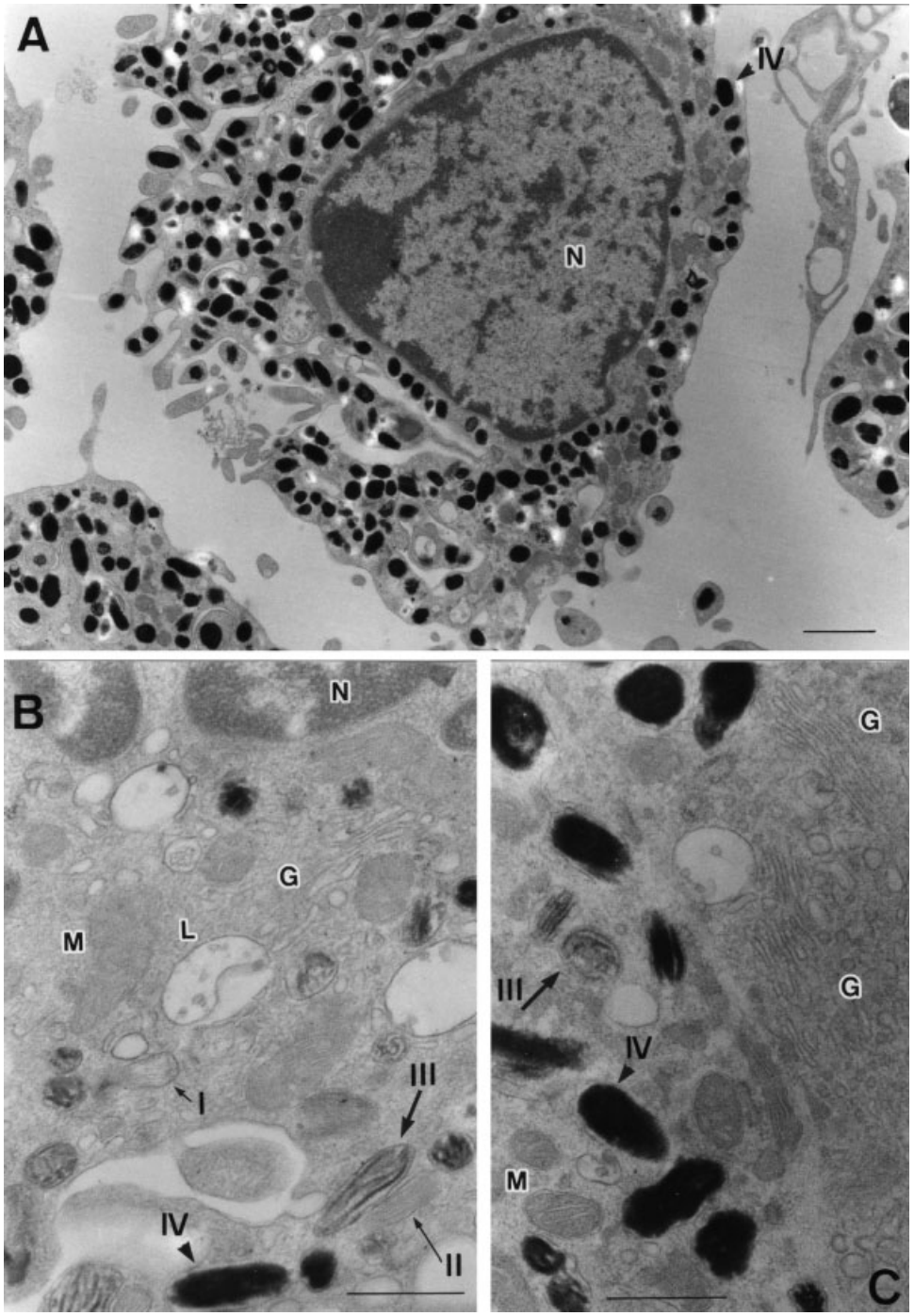

Fig. 2. Electron micrographs of epidermal melanocytes from 0.5-day-old black mice cultured in melanocyte-proliferation medium (MDMD) for 14 days. **A–C:** They possess numerous mature stage IV melanosomes (IV arrowheads) in elliptical morphology. They also possess elliptical stage I (B, I small arrow), II (B, II long arrow), and III (B,C, III large arrows) melanosomes with intraluminal fibrils. Golgi apparatus (G), mitochondria (M), and lysosome (L) are seen. N, nucleus. Scale bar = 1 μm in A, 0.5 μm in B,C.

Electron Microscopic Observations

It has been reported that the epidermal melanocytes cultured in MDMD possess similar biological characteristics to the original neonatal epidermal melanocytes (Hirobe and Abe, 1999, 2000; Hirobe et al., 2002). Therefore, it is conceivable that the observation of the ultrastructure of melanosomes in cultured melanocytes may be valid for studying the effects of the slaty gene on melanosome structures. In cultured black melanocytes derived from 0.5-day-old mice, stage IV melanosomes (Fig. 2A) were predominant. Melanosomes were evenly distributed

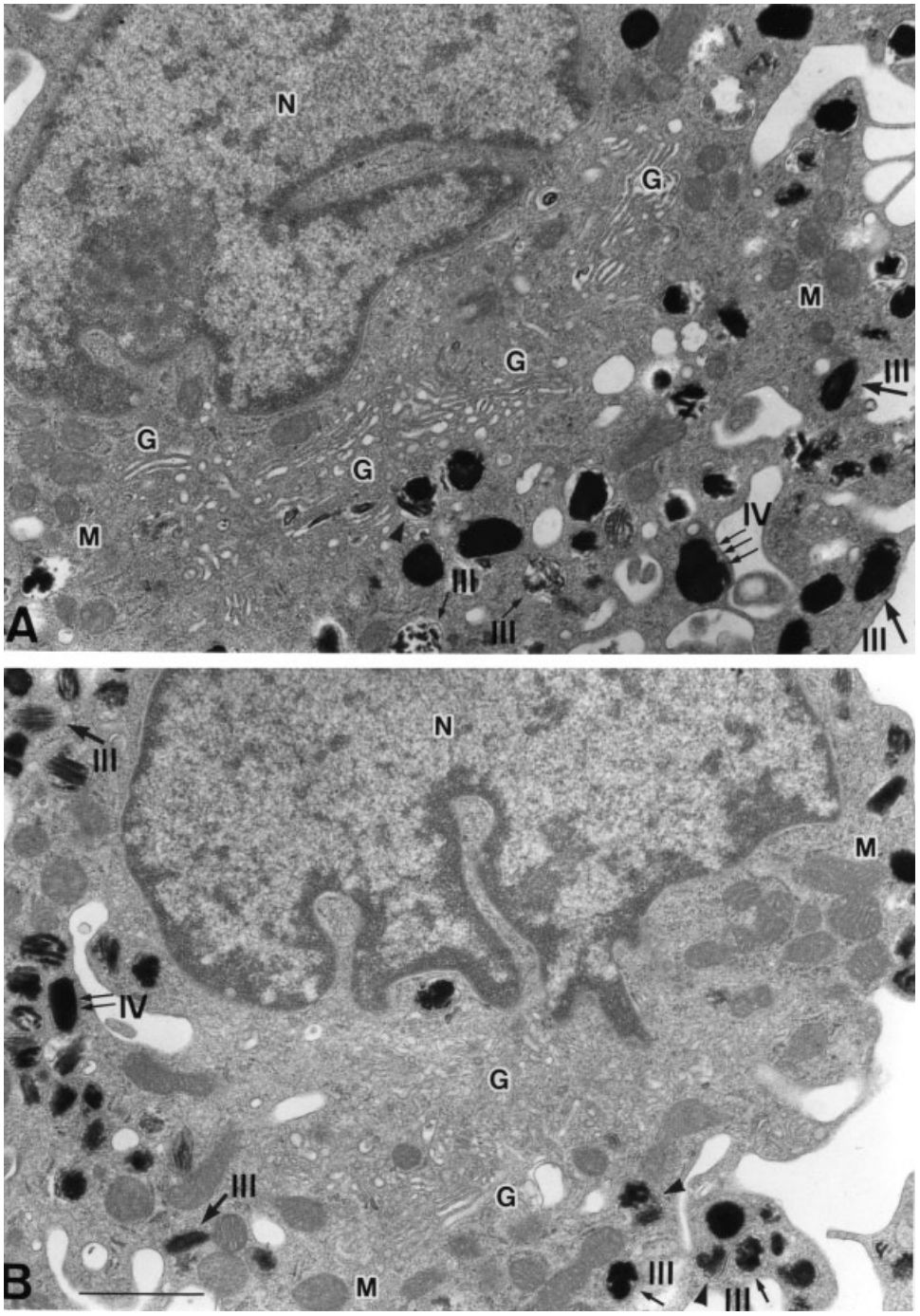

Fig. 3. **A,B:** Electron micrographs of epidermal melanocytes from 0.5-day-old slaty mice cultured in melanocyte-proliferation medium (MDMD) for 14 days. They possess numerous immature stage III (III small arrows) and a few mature stage IV melanosomes (IV triple arrow). Melanosomes are spherical with globular depositions of pigment. They also possess elliptical stage III (III large arrows) and IV (IV double arrow) melanosomes with intraluminal fibrils. A,B: In addition, the mixed-type of the two kinds of melanosomes (arrowheads) is also seen. Numerous Golgi apparatus (G) and mitochondria (M) are seen. N, nucleus. Scale bar = 1 μm.

throughout the cytoplasm. In addition to mature stage IV melanosomes, small numbers of stage I (Fig. 2B), II (Fig. 2B), and III (Fig. 2B,C) melanosomes were also seen. Melanosomes were exclusively eumelanosomes, and their morphology was ellipsoidal or ovoid with intraluminal fibrils (Fig. 2B,C). On the contrary, cultured slaty melanocytes derived from 0.5-day-old mice possessed numerous immature stage III melanosomes (Figs. 3, 4) but a small number of mature stage IV melanosomes (Figs. 3, 4). In addition, a small number of spheri-

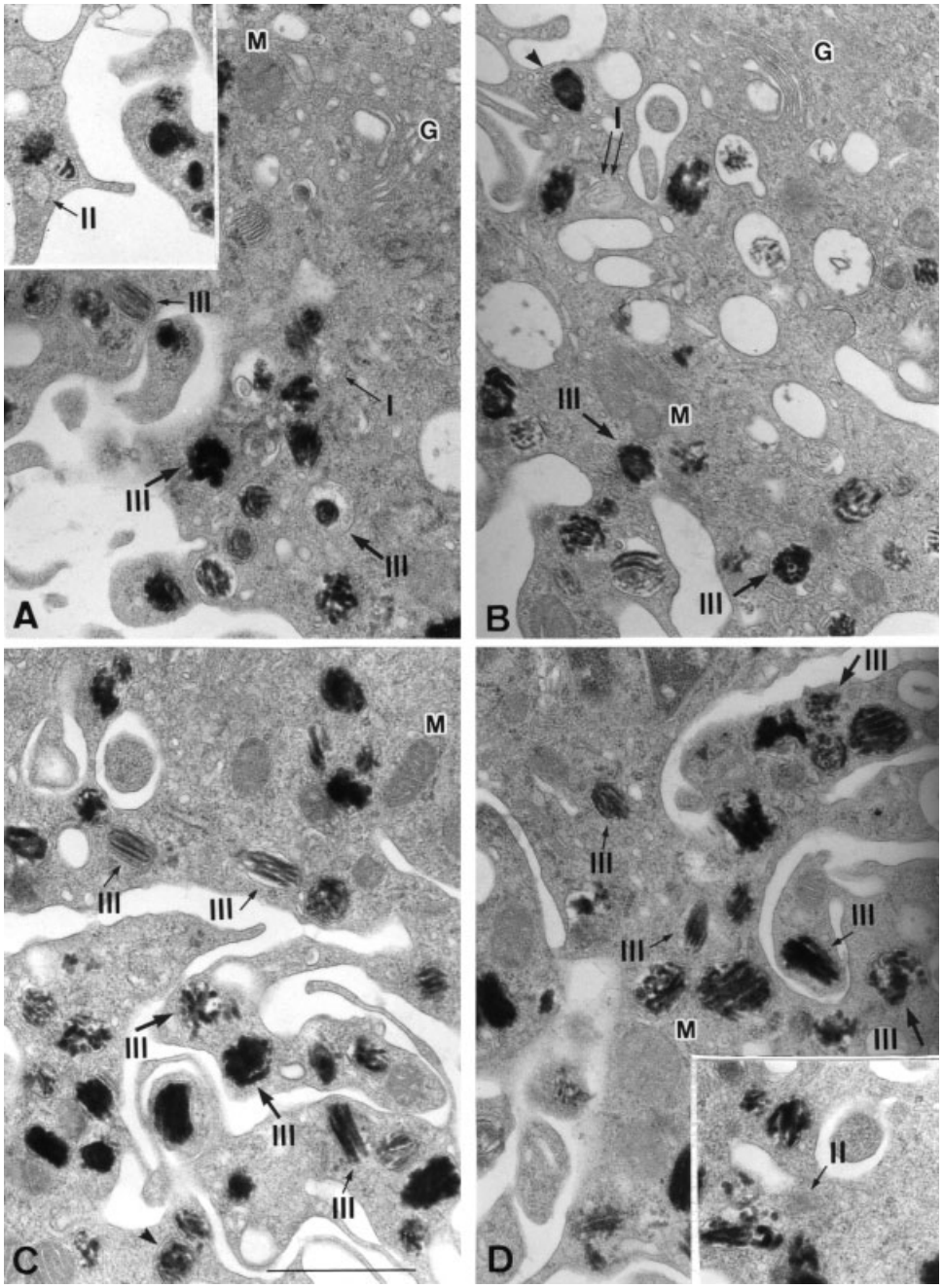

Fig. 4. **A–D:** Electron micrographs of epidermal melanocytes from 0.5-day-old slaty mice cultured in melanocyte-proliferation medium (MDMD) for 14 days. Higher magnification. A: Spherical stage I (I long arrow) and II (insert, II short arrow) melanosomes with many vesicles are also seen. B,D: In contrast, elliptical stage I (B, I double arrow) and II (D insert, II short arrows) melanosomes with intraluminal fibrils are also seen. Numerous spherical stage III melanosomes (A–D, III large arrows) and elliptical stage III melanosomes (A,C,D, III short arrows) with longitudinal depositions of pigment are seen. Moreover, mixed-type melanosomes, which possess two different internal structures (B,C, arrowheads), are seen. Scale bar = 0.4 μm.

cal stage I (Fig. 4A) and II (Fig. 4A insert) melanosomes with small vesicles and spherical stage III (Figs. 3A,B, 4A–D) and IV (Fig. 3A) melanosomes with globular depositions of pigment were also observed. Moreover, elliptical stage I (Fig. 4B) with intraluminal fibrils, stage II (Fig. 4D insert) with completed intraluminal fibrils, stage III (Figs. 3B, 4A,C,D) with pigment depositions at intraluminal fibrils, and stage IV (Fig. 3B) melanosomes with complete depositions of pigment were observed. Moreover, mixed-type melanosomes, including both longitudinal depositions and globular depositions of pigment, were also observed (Fig. 3B).

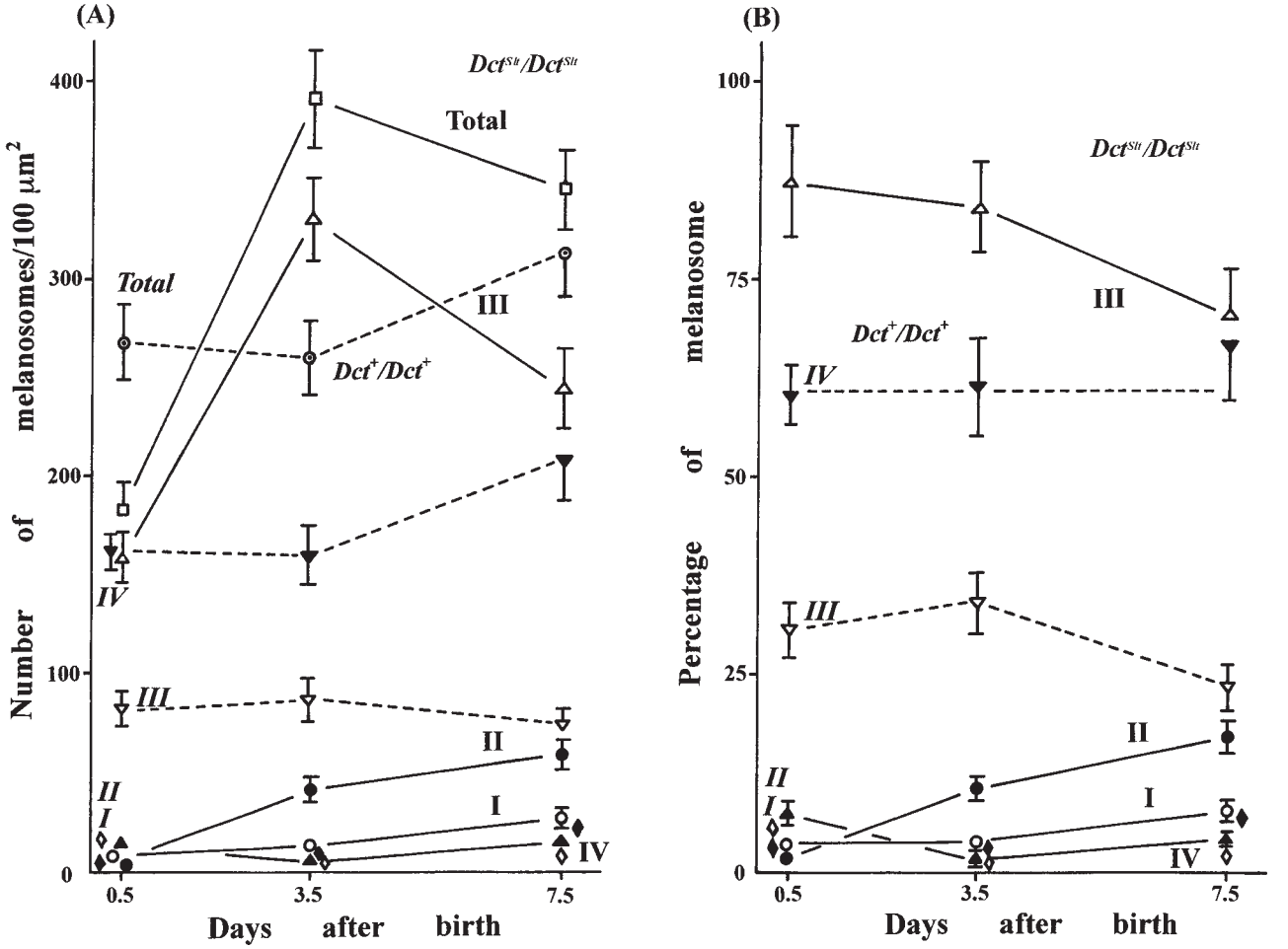

Fig. 5. **A,B:** Number (A) and percentage (B) of stage I (○, slaty; ◇, black), II (●, slaty; ◆, black), III (△, slaty; ▽, black), and IV (▲, slaty; ▼, black) melanosomes in cultured wild-type and slaty melanocytes derived from 0.5-, 3.5-, and 7.5-day-old mice. The number of stage I (I), II (II), III (III), and IV (IV) melanosomes was counted for 35 (black, 0.5 days), 44 (slaty, 0.5 days), 41 (black, 3.5 days), 36 (slaty, 3.5 days), 39 (black, 7.5 days), and 56 (slaty, 7.5 days) figures of nucleated cells. Total number of melanosomes (□, slaty; ●, black) are also calculated per unit area (100 μm^2). The percentage of melanosomes in the total number of melanosomes was calculated. The percentages of stage I and II melanosomes are increased as age advanced, whereas the percentages of stage III and IV melanosomes are decreased in slaty mice. Protocols are the same as in Figure 3.

The number of stage I and II melanosomes in slaty melanocytes was lower than in black melanocytes (Fig. 5A; $P < 0.05$). Moreover, the number of stage IV melanosomes in slaty melanocytes was 12-fold lower than in black melanocytes (Fig. 5A; $P < 0.05$). The total number of melanosomes in slaty melanocytes was also lower than in black melanocytes (Fig. 5A; $P < 0.05$). The percentage of stage IV melanosomes in slaty melanocytes was approximately 8%, whereas that in black melanocytes was approximately 60% (Fig. 5B; $P < 0.05$). In contrast, the percentage of stage III melanosomes in slaty melanocytes was approximately 87%, whereas that in black melanocytes was approximately 31% (Fig. 5B; $P < 0.05$). The percentage of stage I melanosomes in slaty melanocytes was lower than in black melanocytes (Fig. 5B; $P < 0.05$), whereas that of stage II melanosomes did not differ significantly (Fig. 5B). The percentage of elliptical melanosomes in slaty melanocytes was approximately 46%, whereas the percentages of spherical and mixed-type melanosomes were 46% and 8%, respectively (Fig. 6). These values were significantly different from those of black melanocytes (Fig. 6; $P < 0.05$). In mutant melanocytes (Fig. 3), the Golgi apparatus and mitochondria were well-developed and more numerous than in wild-type melanocytes (Fig. 2). On the contrary, the number of lysosomes showed no difference between the two melanocytes.

In cultured epidermal melanocytes derived from 3.5- (Fig. 7A) and 7.5- (Fig. 7B) old-black mice, the numbers and percentages of each stage melanosome showed no notable changes (Fig. 5). On the contrary, in cultured epidermal melanocytes derived from 3.5-day-old slaty mice (Fig. 8A,B), stage II and III melanosomes were increased in number, although stage IV melanosomes were still few. The percentage of stage I or III melanosomes was not changed, whereas that of stage II melanosomes was increased (Fig. 5B). Concerning the type of melanosomes, elliptical stage II and III melanosomes

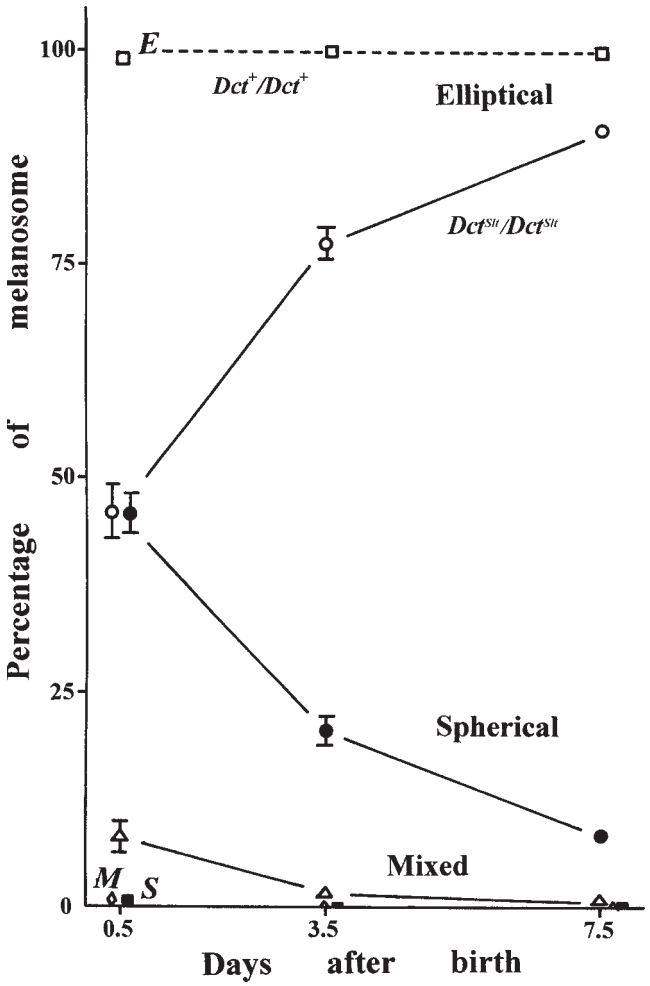

Fig. 6. Percentage of elliptical (○, slaty; □, black), spherical (●, slaty; ■, black), and mixed-type (△, slaty; ◇, black) melanosomes in cultured black and slaty melanocytes derived from 0.5-, 3.5-, and 7.5-day-old mice. Spherical melanosomes and mixed-type melanosomes are decreased as age advanced, whereas elliptical melanosomes are increased in slaty melanocytes. Protocols are the same as in Figure 3.

were increased dramatically (Figs. 6, 8). On the contrary, spherical melanosomes and mixed type melanosomes were dramatically decreased (Figs. 6, 8). In addition to these changes, the Golgi apparatus was also decreased in number (Figs. 8, 9). On the contrary, mitochondria (Fig. 9B) and lysosomes (Fig. 9C) showed no notable changes. In cultured epidermal melanocytes derived from 7.5-day-old slaty mice (Fig. 10), the number of stage I and II melanosomes were further increased, whereas stage III melanosomes were decreased (Fig. 5A,B). Stage IV melanosomes were still few (Fig. 10A). Concerning the type of melanosomes, elliptical melanosomes were further increased, whereas spherical melanosomes and mixed-type melanosomes were further decreased (Figs. 6, 10). Moreover, mitochondria of slaty melanocytes were increased in number from 3.5 to 7.5 days (Fig. 9B). This increase is statistically significant ($P < 0.05$). In contrast, the Golgi apparatus (Fig. 9A) and lysosomes (Fig. 9C) of slaty melanocytes were not changed significantly from 3.5 to 7.5 days.

DISCUSSION

The process of biogenesis of eumelanosomes of black mice (Hirobe and Takeuchi, 1978) as well as pheomelanosomes of recessive yellow (Tamate and Takeuchi, 1984) and lethal yellow (Jimbow et al., 1979; Tamate and Takeuchi, 1981) mice has been studied using electron microscopy, and the discrimination between stage I, II, III, and IV melanosomes (Fitzpatrick et al., 1969) has also been performed. The ideal way to investigate the effects of the slaty gene on the biogenesis of melanosomes may be the observation of epidermal melanocytes without culture. However, it is difficult to observe numerous epidermal melanocytes, because of their limited number in the epidermis. The observation of cultured epidermal melanocytes may be a good way to examine the effects of the slaty gene, because the structures of melanosomes, the Golgi apparatus, and mitochondria of cultured melanocytes are comparable to those of melanocytes without culture (Hirobe and Abe, 1999, 2000; Hirobe et al., 2002) and, in addition, numerous melanosomes can be observed from numerous cultured melanocytes.

Our present study reveals that the *Dct*^{slt} mutation blocks the melanosome biogenesis at stage III. The difference between stage III and IV melanosomes may be merely the content of pigment depositions. The results that slaty melanocytes possessed numerous stage III melanosomes but an extremely low number of stage IV melanosomes suggest that DCT activity may be required to maximize melanin depositions within melanosomes. The inhibition of melanogenesis in cultured slaty melanocytes may be due to the inhibition of melanosome maturation. It is reported that the injection of α-melanocyte stimulating hormone (MSH) or DBcAMP stimulates the differentiation of epidermal melanocytes in black (C57BL/10JHir-*Dct*$^{+}$/*Dct*$^{+}$) mice (Hirobe and Takeuchi, 1977). The differentiation stimulated by α-MSH is associated with the increase in the number and percentage of stage IV melanosomes (Hirobe and Takeuchi, 1978). The results that black melanocytes cultured in MDMD (including DBcAMP) contained numerous stage IV melanosomes in this study agree well with the reports of mouse epidermal melanocytes without culture (Hirobe and Takeuchi, 1977, 1978). We reported that stages III and IV melanosomes increased in the epidermal melanocytes of black (C57BL/10JHir-*Dct*$^{+}$/*Dct*$^{+}$) mice from 3 to 6 days in vivo (Hirobe and Takeuchi, 1978). The epidermal melanocytes possessed exclusively elliptical stage III and IV melanosomes. The present results of cultured black melanocytes from 3.5- and 7.5-day-old mice are consistent with our previous in vivo results. Moreover, the results that slaty melanocytes contained a limited number of stage IV melanosomes in this study suggest that the slaty gene inhibits the maturation of stage IV melanosomes.

In this study, the number of stage III melanosomes in slaty melanocytes was increased from 0.5- to 3.5-day-old mice, although the number of mature stage IV melanosomes failed to increase. Therefore, it is conceivable that the slaty mutation may block the melanosome biogenesis at stage III. Spherical melanosomes with globular depositions of pigment that are predominant at slaty melanocytes derived from 0.5-day-old mice were gradually decreased in number as age advanced, whereas elliptical melanosomes with intraluminal fibrils were gradually increased in number. In this

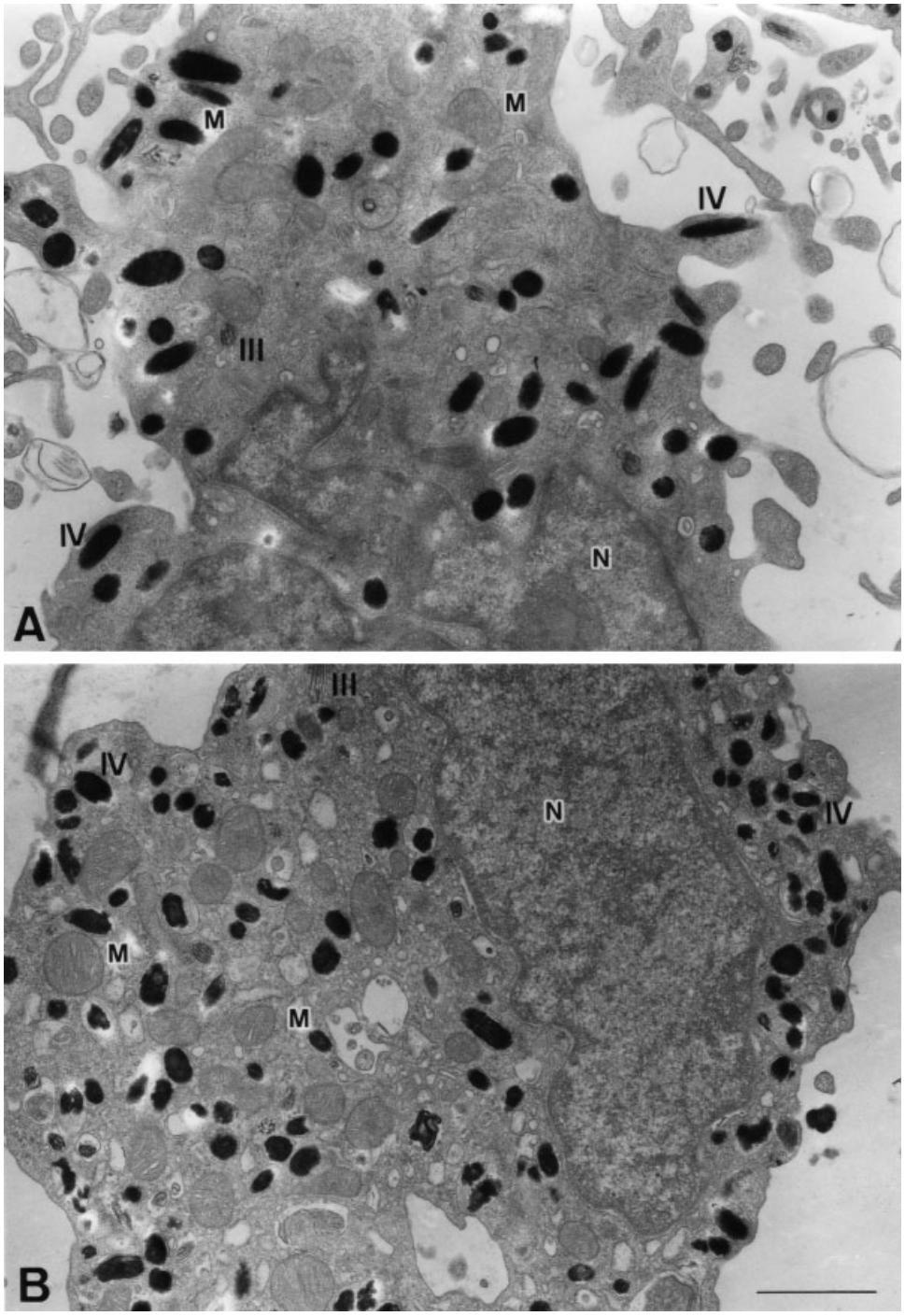

Fig. 7. **A,B:** Electron micrographs of epidermal melanocytes from 3.5- (A) and 7.5- (B) day-old black mice cultured in melanocyte-proliferation medium (MDMD) for 14 days. They possess numerous stage III (III) and IV (IV) elliptical melanosomes. M, mitochondria; N, nucleus. Scale bar = 1 μm.

study, cultured wild-type melanocytes derived from 0.5-day-old mice possessed mostly elliptical eumelanosomes at stages III (31%) and IV (60%). Therefore, it is reasonable to think that the slaty mutation affects the internal structure of melanosomes in addition to the depositions of pigment in a developmental stage-specific manner. The lowered DCT activity in slaty melanocytes may be involved in regulating the development of internal structure of melanosomes in addition to melanin depositions.

Costin et al. (2005) reported that, in immortalized slaty dermal melanocytes derived from 1-day-old C57BL/

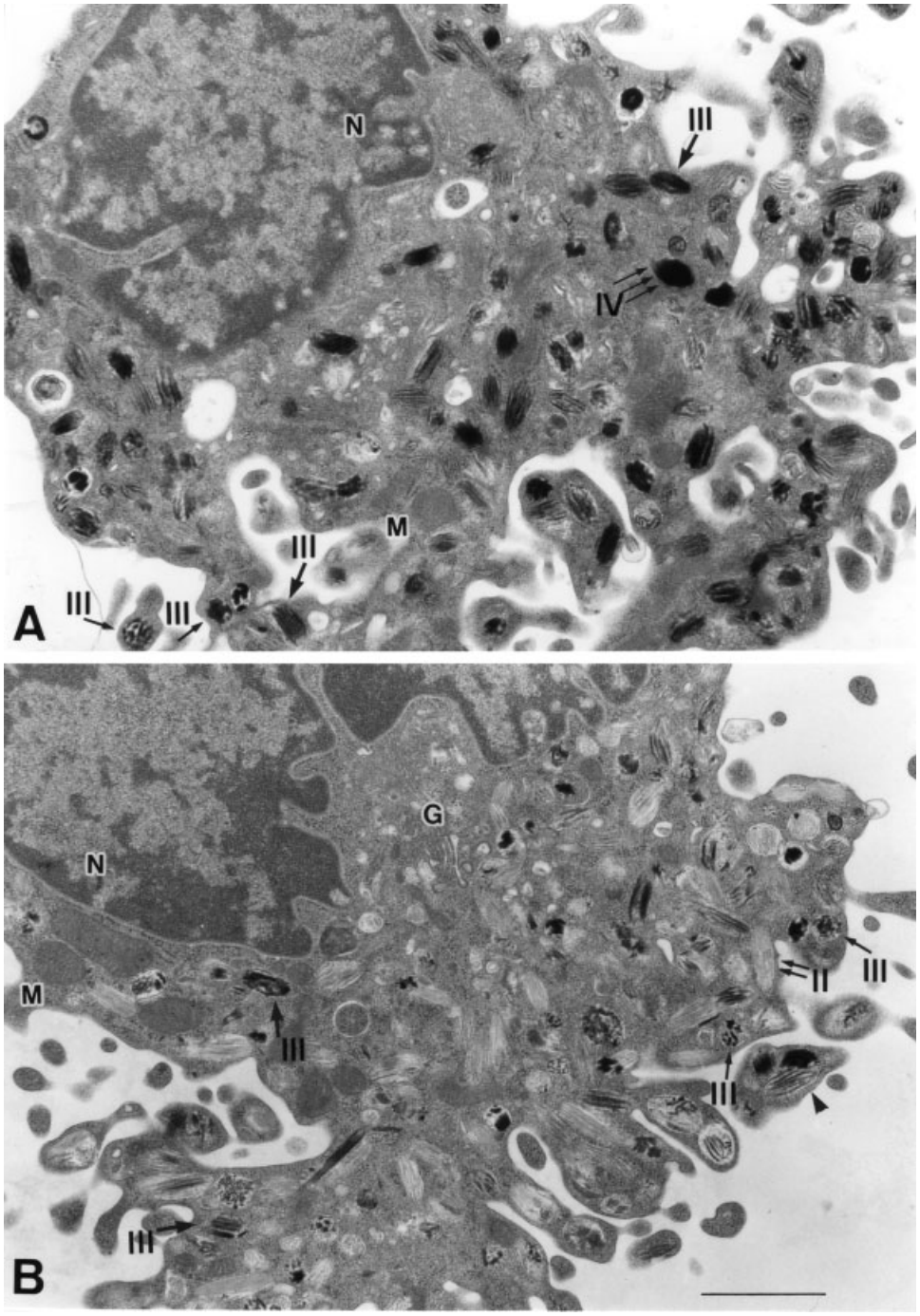

Fig. 8. Electron micrographs of epidermal melanocytes from 3.5-day-old slaty mice cultured in melanocyte-proliferation medium (MDMD) for 14 days. **A,B:** They possess numerous stage II (II double arrow, B), III elliptical melanosomes (III large arrows), and a small number of mature stage IV elliptical melanosomes (IV triple arrow), in addition to stage III spherical melanosomes (III small arrows; A,B). B: Mixed-type melanosome (arrowhead) is also seen. The number of spherical melanosomes is lower than in 0.5-day-old mice (Fig. 3). The Golgi apparatus (G) is decreased in size, whereas numerous mitochondria (M) are seen. N, nucleus. Scale bar = 1 μm.

6J-Dct^{Slt}/Dct^{Slt} mice, the content of 4-amino-3-hydroxyphenylalanine (4-AHP; Wakamatsu et al., 2002), which is a specific degradation product of pheomelanin, was increased (fivefold increase) compared with immortalized wild-type dermal melanocytes derived from 1-day-old C57BL/6J-Dct^{+}/Dct^{+} mice. Our previous study (Hirobe et al., 2006) also showed that the content of 4-AHP in homogenates of dermis derived from 3.5-day-old slaty mice as well as in culture medium of epidermal melanocytes derived from 7.5-day-old mice was greatly

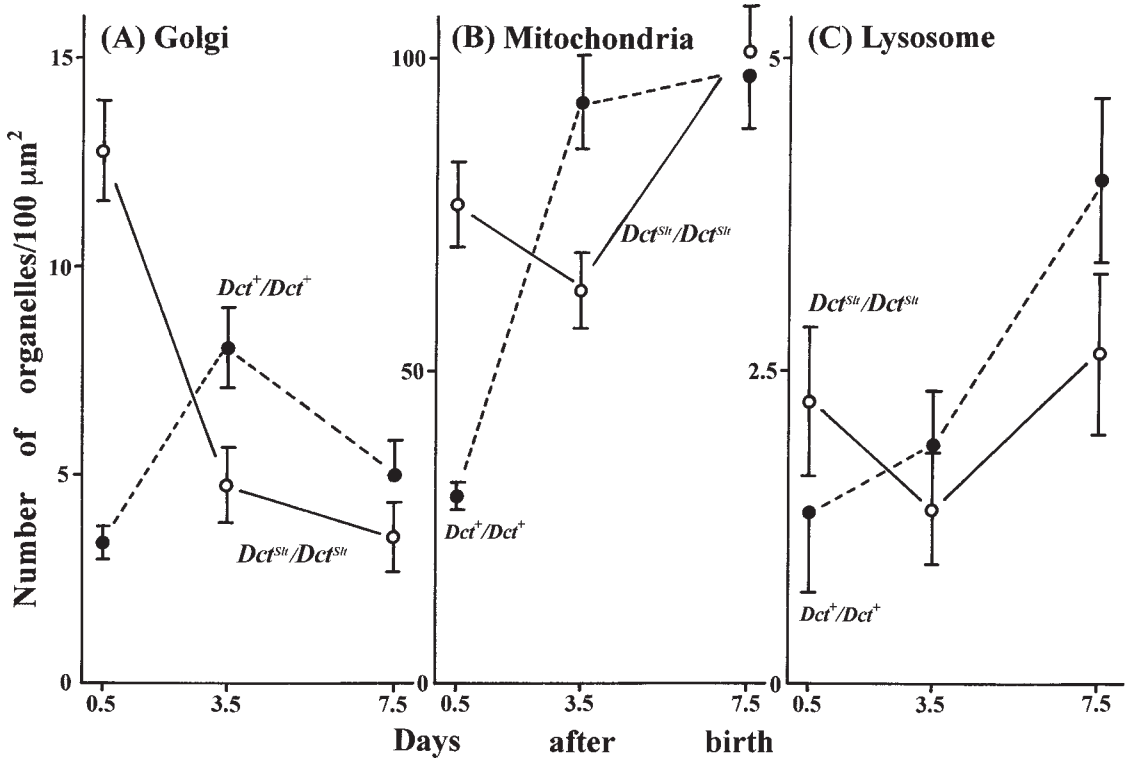

Fig. 9. **A–C:** Changes in the number of the Golgi apparatus (A), mitochondria (B), and lysosomes (C) in cultured black and slaty melanocytes derived from 0.5-, 3.5-, and 7.5-day-old mice. The number of Golgi apparatus is decreased, whereas that of mitochondria is increased after 3.5 days in slaty melanocytes. In contrast, lysosomes show no notable change. Protocols are the same as in Figure 5.

increased. However, Costin et al. (2005) reported that immortalized slaty dermal melanocytes derived from 1-day-old mice possessed mostly stage II and III eumelanosomes. The present study demonstrated that slaty melanocytes possessed spherical melanosomes with globular depositions of pigment and mixed-type melanosomes in addition to elliptical melanosomes with intraluminal fibrils. However, our previous study (Hirobe et al., 2006) showed that slaty melanocytes did not possess greater amount of 4-AHP than wild-type melanocytes. Therefore, it is conceivable that slaty melanocytes are capable of synthesizing pheomelanin, but are incapable of retaining pheomelanin in melanosomes.

Our previous study (Hirobe et al., 2006) showed that the content of pyrrole-2,3,5-tricarboxylic acid (PTCA; Ito and Fujita, 1985), a specific degradation product of eumelanin, was decreased in cultured slaty melanocytes. The lowered PTCA content may be the result of the dramatic decrease in the number of stage IV melanosomes as revealed in this study. The reduced DCT activity may inhibit the maturation of stage IV melanosome, and, in consequence, the eumelanin content may be greatly reduced.

The slaty melanocytes derived from 0.5-day-old mice were shown to possess much greater number of the Golgi apparatus than black melanocytes, and the number of the Golgi apparatus was dramatically decreased as age advanced. One possibility exists that the abundance of the Golgi apparatus induces the formation of spherical melanosomes. The Golgi apparatus plays an important role in the melanosome formation (Imokawa and Mishima, 1981; Hirobe, 1982; Kushimoto et al., 2001, 2003; Dell'Angelica, 2003). Moreover, the Golgi apparatus decreases in association with the increase of stage IV eumelanosomes during the normal and α-MSH–induced differentiation in epidermal melanocytes of C57BL/10JHir-Dct^{+}/Dct^{+} mice (Hirobe and Takeuchi, 1978). These results suggest the possibility that the stimulation of eumelanosome biogenesis is associated with the decrease of the Golgi apparatus. Because in slaty melanocytes, the maturation of melanosomes is greatly inhibited, the decrease in the Golgi apparatus may be inhibited compared with black melanocytes. Thus, the lowered DCT activity may increase the biogenesis of the Golgi apparatus. Although mitochondria in cultured slaty melanocytes were increased from 3.5 days to 7.5 days in the present study, it remains to be solved whether mitochondria are involved in regulating the biogenesis of melanosomes in slaty melanocytes. In black melanocytes, mitochondria increased from 0.5- to 3.5-day-old mice and showed a similar level as those of slaty melanocytes from 7.5-day-old mice in this study.

Garcia and Szabo (1983) reported that, in embryonic chick retinal pigment epithelia, the type of melanosomes changed from spherical to elliptical by culturing them in a medium containing chick embryo extracts and fetal bovine serum. The spherical melanosomes observed in slaty melanocytes in this study are very similar to the spherical melanosomes in embryonic chick retinal pigment epithelia reported by them. Their results as well as the present findings suggest the possibility that the ultrastructure of melanosomes can be modulated by genetic factors and culture conditions.

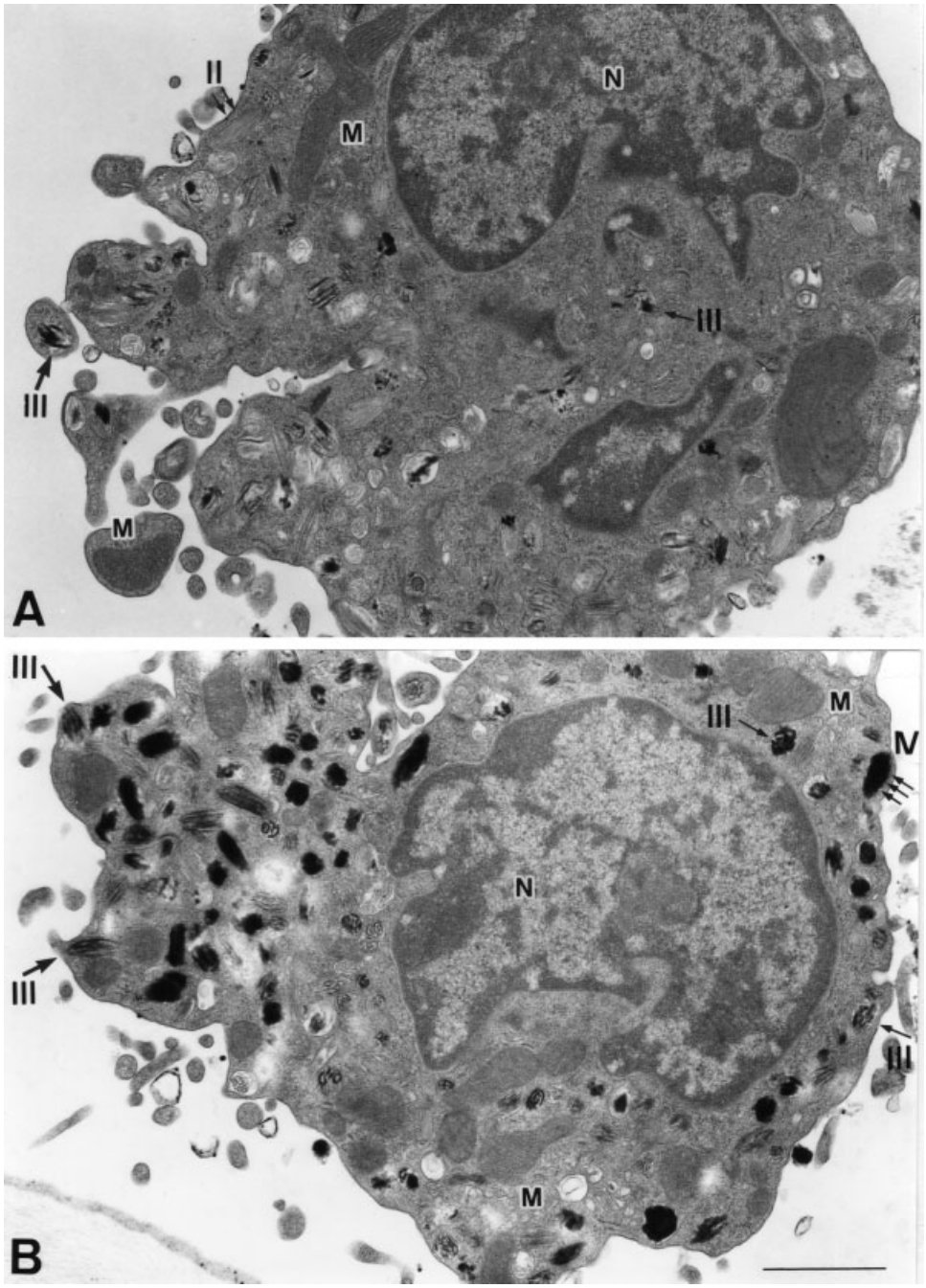

Fig. 10. **A,B:** Electron micrographs of epidermal melanocytes from 7.5-day-old slaty mice. They possess numerous stage II (II double arrow, A), stage III (III large arrows, A,B) elliptical melanosomes and a small number of mature stage IV elliptical melanosomes (IV triple arrow, B) in addition to stage III spherical melanosomes (III small arrows, A,B). The number of spherical melanosomes is decreased. A,B: The Golgi apparatus is not seen in these micrographs, whereas numerous mitochondria (M) are seen. N, nucleus. Scale bar = 1 μm.

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