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Ultrastructural Observations on DMBA-Induced Dermal Hyperpigmentation and Blue Nevus-Like Tumors in the Mongolian Gerbil (40797)[1]

WALTER C. QUEVEDO, JR., ROBERT D. FLEISCHMANN, JACOB DYCKMAN, KOWICHI JIMBOW, AND THERESA C. BIENIEKI

Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912, and Department of Dermatology, Sapporo Medical College, Sapporo, Japan

Topical applications of 7,12-dimethylbenz(*a*)anthracene (DMBA) to the dorsal trunk skin of Mongolian gerbils (*Meriones unguiculatus*) elicit blue nevus-like melanotic tumors and hyperpigmentation of the epidermis and dermis (1). At the light microscope level, the blue nevus-like tumors of the gerbil resemble those induced by DMBA in Syrian golden hamsters and black mice (2, 3).

An earlier study examined the ultrastructure of gerbil epidermis hyperpigmented by topical applications of DMBA (4). The ultrastructure of the DMBA-induced dermal hyperpigmentation and melanotic blue nevus-like tumors forms the basis for this report. Dermal hyperpigmentation was examined in skin treated with DMBA alone or DMBA and croton oil. The considerable variation in the morphology of melanosomes encountered suggests that melanin-containing granules are synthesized and degraded via several different pathways in the carcinogen-treated dermis.

Materials and methods. Electron microscopic observations were made on the dorsal hyperpigmented dermis of nine gerbils which were 3–6 months of age when treatments were initiated. In four of these animals, blue nevus-like tumors, ranging in size from 2 to 10 mm in greatest diameter, were also examined.

Two male and two female gerbils were treated once weekly with 1 ml of 0.1% DMBA (Eastman Organic Chemicals) in acetone for 12 weeks. The DMBA solution was released on the shaved dorsum of each animal from a calibrated syringe. The animals were sacrificed and tumors removed from the dorsum approximately 1 to 5 months after the last treatment with DMBA. In each case, samples of the tumor and hyperpigmented dermis were obtained for electron microscopy.

Early stages in dermal hyperpigmentation were examined in three female gerbils treated with 1 ml of 0.1% DMBA in acetone once weekly for 3 weeks and sacrificed 1 week later. Dermal hyperpigmentation was also examined in one male and one female gerbil which had received 1 ml of 0.1% DMBA in acetone once a week for 5 weeks, followed by thrice weekly applications of 1 ml of 1% croton oil in acetone for 27 weeks.

Skin and tumor specimens were fixed in paraformaldehyde–glutaraldehyde, postfixed in osmium tetroxide, and embedded either in Spurr low viscosity epoxy resin or epon-araldite (5, 6). Sections were made on a Porter–Blum MT-2 ultramicrotome (Sorvall), stained with uranyl acetate and lead citrate, and examined in a Zeiss EM 9S-2 electron microscope.

Thick sections were stained with toluidine blue and examined by light microscopy. For a few skin and tumor specimens, postfixation in osmium tetroxide was omitted so that the natural electron opacity of melanosomes could be observed. The unstained sections were examined by light and electron microscopy.

Results. Light microscopy. The blue nevus-like tumors consisted of dense aggregates of variably melanized dermal cells which often produced a bulging of the overlying epidermis (Fig. 7). The latter was frequently characterized by areas of irregular acanthosis, hyperkeratosis and numerous melanotic melanocytes along with variable amounts of melanin within keratinocytes. Most of the dermal tumor

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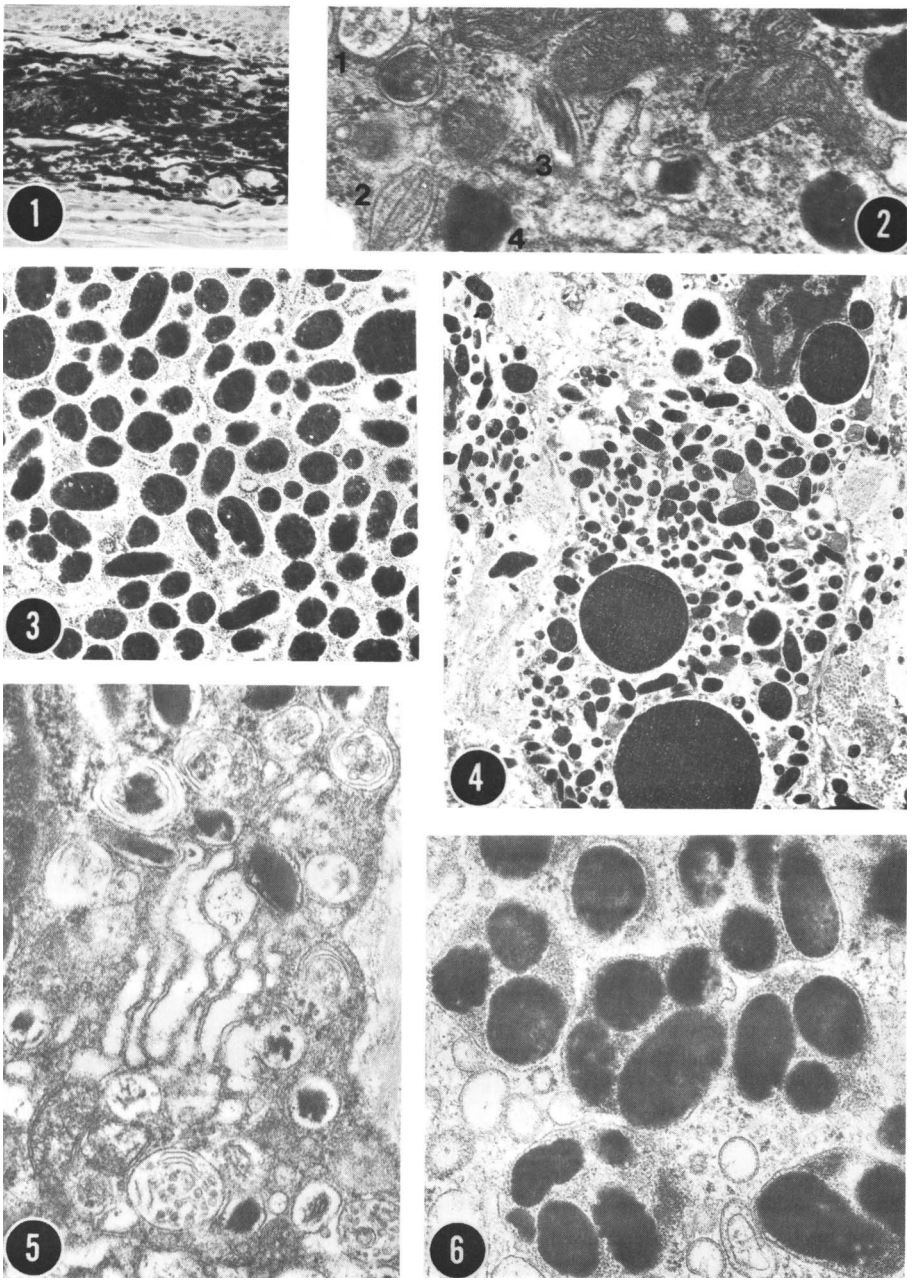

FIG. 1. Light micrograph demonstrating marked dermal hyperpigmentation of skin treated with DMBA-croton oil (×92). (Orientation for electron micrographs of Figs. 2–6.)

FIG. 2. Melanosomes at various stages (I–IV) of formation within a DMBA-treated dermal melanocyte. The developing melanosomes are round to elliptical and characterized by lamellae or filaments upon which melanin is deposited (×29,450).

FIG. 3. Portion of the cytoplasm of a DMBA-treated dermal melanocyte which contains only mature elliptical (Stage IV) melanosomes (×14,570).

FIG. 4. Macromelanosomes within dermal melanocytes of DMBA-treated skin. Mature melanosomes of normal and intermediate size are also present in large numbers (×5580).

cells were characterized by a high nuclear/cytoplasmic ratio, finely dispersed pigment, and a fusiform to polyhedral shape. Some cells appeared to be macrophages based on their coarser melanin pigment, polyhedral shape, abundant cytoplasm, and relatively small, eccentrically placed nuclei. Some pigmented cells were intermediate in morphology. Although mitotic activity was evident in the melanotic cells of some tumors, cell atypism in the form of nuclear enlargement and hyperchromatism was present but not pronounced. Reticulin fibers surrounded small and, less frequently, large groups of tumor cells. Some tumors were more sharply demarcated than others from the surrounding dermis and infiltrations of the adipose layer were noted which occasionally extended down to the striated muscle layer, suggesting malignant invasion. Infrequently tumor cells eroded the dermoepidermal junction and entered the overlying epidermis. The tumors did not appear to metastasize or obviously influence the survival of their hosts during the period of observation. Melanotic macrophages were found in the lymph nodes of some animals and smaller numbers of dendritic cells resembling melanocytes were found scattered among the lymphoid cells. The scattered isolated melanocytic cells did not fit the typical pattern of metastatic malignant melanoma.

In contrast to the localized nature of the blue nevus-like tumors, the dermal hyperpigmentation was diffuse extending over broad areas of the skin. It appeared to result from a marked increase in the numbers of normal melanogenically active dendritic melanocytes surrounding hair follicles and extending between them forming an interlacing network in the papillary and reticular dermis (1, 7) (Fig. 1). Melanotic macrophage-like cells were also found but in smaller numbers than the melanocytes. Whereas the tumor cells were frequently of epithelioid morphology and often tightly packed with little or no intervening stroma, the dermal melanocytes were more highly dendritic and loosely arranged among the fibrous elements of the dermis.

Electron microscopy. Hyperpigmented dermis. Stages in melanosome development were designated following the system described by Fitzpatrick *et al.* (8): Stages I and II characterized unmelanized early melanosomes, Stage III incompletely melanized melanosomes, and Stage IV fully mature, uniformly melanized melanosomes (Fig. 2). Some dermal melanocytes (Figs. 3, 4) contained large numbers of mature (Stage IV) melanosomes (maximum size ca. $0.3-0.4 \times 0.8-1.0\ \mu m$) with no evidence of formative stages (i.e., Stages I–III). Small electron-lucent bodies, about 40 nm in diameter, were evident in many of the melanosomes. Each melanosome was limited by a membrane and grouping of melanosomes within autophagosomes was rarely encountered. In other dermal melanocytes, Stages I–III melanosomes were found along with the mature ones (Fig. 2). The immature melanosomes of the dermal melanocytes were characterized by a filamentous matrix which was the initial site of melanin deposition. Within Stage II melanosomes, the filaments were approximately aligned in parallel and usually exhibited a periodicity of structure which took the form of striations oriented at right angles to the long axis of each filament. The deposition of melanin on the aligned filaments signaled the transition from Stage II to Stage III. Round, membrane-limited vacuoles containing myelin-like structures and/or tangled networks of nonaligned fine filaments and several microvesicles have been tentatively identified as Stage I melanosomes (Figs. 2, 5) (9, 10). Apparently, melanin synthesis occurred prematurely in some cases, for occasional dermal melanocytes contained, in addition to typical (i.e., amelanotic) Stage I melanosomes, comparable granules with melanin-like

FIG. 5. Stage I multivesicular-fibrillar melanosomes in dermal melanocytes of skin treated with DMBA-croton oil. Some granules contain myelin-like structures and possibly melanin (×29,450).

FIG. 6. Compound melanosomes (secondary lysosomes) within a dermal macrophage of DMBA-croton oil treated skin (×29,450).

dense material deposited on the internal disorganized array of filaments and microvesicles (Fig. 5).

In one specimen of hyperpigmented dermis, unusually large elliptical to round electron dense granules (maximum diameter ca. 5–6 μm) were found within dermal melanocytes which also contained elliptical melanosomes of normal size (Fig. 4). The giant pigment granules exhibited discrete electron-lucent bodies and closely approximated in appearance the macromelanosomes previously described in the pigmented macules of neurofibromatosis and nevus spilus (11, 12). Within the melanocytes containing macromelanosomes, the smaller melanosomes were round to elliptical in shape and varied considerably in size. Irrespective of size the melanosomes were fully melanized (Stage IV). Dermal melanocytes were frequently surrounded by a basal lamina which was absent from macrophages.

Within the macrophage-like melanin-bearing dermal cells, melanosomes were usually arranged in groups of two or more immersed in a particulate material which was surrounded by a limiting membrane (Fig. 6). Occasional single melanosomes were found within comparable particle-studded membrane-bound vacuoles. The membrane-limited aggregates of melanosomes closely resembled in morphology the melanosome-containing secondary lysosomes (melanosome complexes) found within keratinocytes (8).

Blue nevus-like tumors. As in the dermal melanocytes, the cells of the blue nevus-like tumors (Fig. 7) contained mainly Stage IV melanosomes which ranged from ellipsoidal (maximum size ca. 0.3–0.4 × 1.0–1.3 μm) to round (maximum diameter ca. 0.7–0.9 μm) in shape (Fig. 8). The presence of typical Stage II and Stage III melanosomes indicated that at least some of the melanocytes in the tumor paralleled those of hyperpigmented dermal melanocytes in their mode of melanosome synthesis (Figs. 9, 10). As in the case of dermal melanocytes, elliptical membrane-bound vacuoles containing microvesicles and nonaligned filaments have been found within the tumors and tentatively designated as Stage I melanosomes (Fig. 10). Occasionally, prominent microvesicles have been found in Stage II melanosomes of the melanotic tumors.

In some tumor cells clearly abnormal melanosomes were formed. Melanosomes were observed in which filaments and/or vesicles contained deposits of electron-dense material which suggested that melanin was synthesized in the absence of a well-defined filamentous matrix (Figs. 9, 11). In some cases it was difficult to determine whether the small melanizing elements were filamentous or vesicular. The independently melanizing elements apparently coalesced to form more typical-appearing round (Stage IV) melanosomes. Some mature round/ellipsoidal melanosomes displayed very irregular deposits of melanin within their interior. Small non-melanized microvesicles were found within a number of melanosomes at various stages of melanization. Thus, there is the indication that within the blue nevus-like tumors, melanosome synthesis may take place (i) in the standard manner of filaments or lamellae aligning to form a matrix within a membrane-limited vacuole, and (ii) by the aggregation of melanized filaments and/or vesicular bodies which are cemented together by melanin depositied on their surfaces.

Melanosomes were found either as singles or in membrane-bound groups of varying size within the tumor. In some tumor cells, the melanosomes were arranged solely in large secondary lysosomes and showed evidence of partial degradation (Fig. 9). In ultrastructure the melanin-bearing secondary lysosomes appeared to be autophagic in origin rather than heterophagic. Based on the continuum of tumor cells from those with no melanin-bearing secondary lysosomes to those in which all melanosomes were grouped within their secondary lysosomes, it is tentatively concluded that essentially all pigmented cells within the tumors represent a single cell type, i.e., melanocyte, at various stages of differentiation and deterioration.

Discussion. The dermal melanocytes and blue nevus-like tumor cells of carcinogen-treated gerbil skin show considerable ver-

satility in the synthesis of melanosomes. Paralleling the epidermal melanocytes of DMBA-treated gerbils, the dermal melanocytes and tumor cells synthesize typical ellipsoidal melanosomes in which parallel arrays of filaments or lamellae serve as the matrix upon which melanin is deposited.

In addition, occasional DMBA-treated dermal melanocytes synthesize giant, (usually round) electron-dense bodies which closely correspond to the macromelanosomes described in several human pigmentary conditions. Although formative stages were absent, the presence of electron-lucent bodies within the gerbil "macromelanosomes" suggests that they, paralleling the macromelanosomes of man, contain a matrix of numerous microvesicles upon which melanin is deposited (11–15). Electron-lucent bodies are also found in the more normal-sized ellipsoidal melanosomes. The role played by microvesicles in the formation of normal melanosomes and macromelanosomes is still uncertain (12).

Still another possible pathway of melanosome synthesis is regularly observed in melanotic tumor cells and less frequently and less clearly found in dermal melanocytes. In this case, vesicular and irregular fibrillar elements of Stage I melanosomes melanize prematurely and coalesce to produce a round melanosome of normal pigmentation and size. At their earliest stages these melanosomes are similar in morphology to pigment-free multivesicular bodies found within the same cells. A number of authors have designated multivesicular-like bodies as precursors or early stages of melanosomes in a variety of organisms ranging from fish to mammals (9, 10, 16).

The pattern of maturation of the small round multivesicular-fibrillar melanosome is quite distinct from that previously described for macromelanosomes (11, 12). Although repeated applications of croton oil do not stimulate the production of blue nevus-like tumors in the gerbil, they do elicit marked hyperpigmentation of the dermis (1). The irregularly melanized multivesicular-fibrillar Stage I-like melanosomes were found in gerbils which received both DMBA and croton oil. A subsequent study will determine whether these unusual organelles were attributable to the DMBA, the DMBA plus croton oil, or the croton oil.

Although ellipsoidal filamentous melanosomes have been observed in the blue nevus-like tumors of black mice and Syrian golden hamsters (17, 18), neither macromelanosomes nor melanin deposition within multivesicular-fibrillar premelanosomes has been reported previously.

Ultimately, the melanosomes produced by tumor cells in the gerbil are grouped within autophagosomes and degraded. Formative stages of melanosomes as well as autophagosomes have been observed within the same tumor cell. In overall dynamics, the melanotic tumors of the gerbil correspond to the Harding–Passey mouse melanoma where Seiji and Otaki (19) have described three stages in melanoma cell differentiation: (i) melanosome production, (ii) melanosome production and autophagocytosis, and (iii) melanosome-bearing (postmelanization, solely autophagic). Novikoff *et al.* (20) have concluded that compound melanosomes in B-16 and Harding–Passey melanoma cells are in reality secondary lysosomes associated with autophagocytosis of melanosomes. This conflicts with earlier reports of large numbers of macrophages as major ingredients of the Harding–Passey melanoma and melanotic tumors in swine (21, 22). The marked autophagic activity may be preliminary to melanocyte death (23). Recently, Kleihues *et al.* (24) have described the selective induction of benign melanomas in gerbils following postnatal administration of *N*-ethyl-*N*-nitrosourea. The tumors exhibited a long latency period of approximately 2.5 years and were almost entirely restricted to the relatively hairless extremities. Since few details were provided on their general histology and ultrastructure, their relationship to the DMBA-induced blue nevus-like tumors described herein remains to be determined. In addition, the precise origin of the blue nevus-like tumors is not known. Possible sources might be the epidermal or dermal melanocytes or neural elements associated with hair follicles (1).

Summary. Electron microscopic observations were made on the dermal hyper-

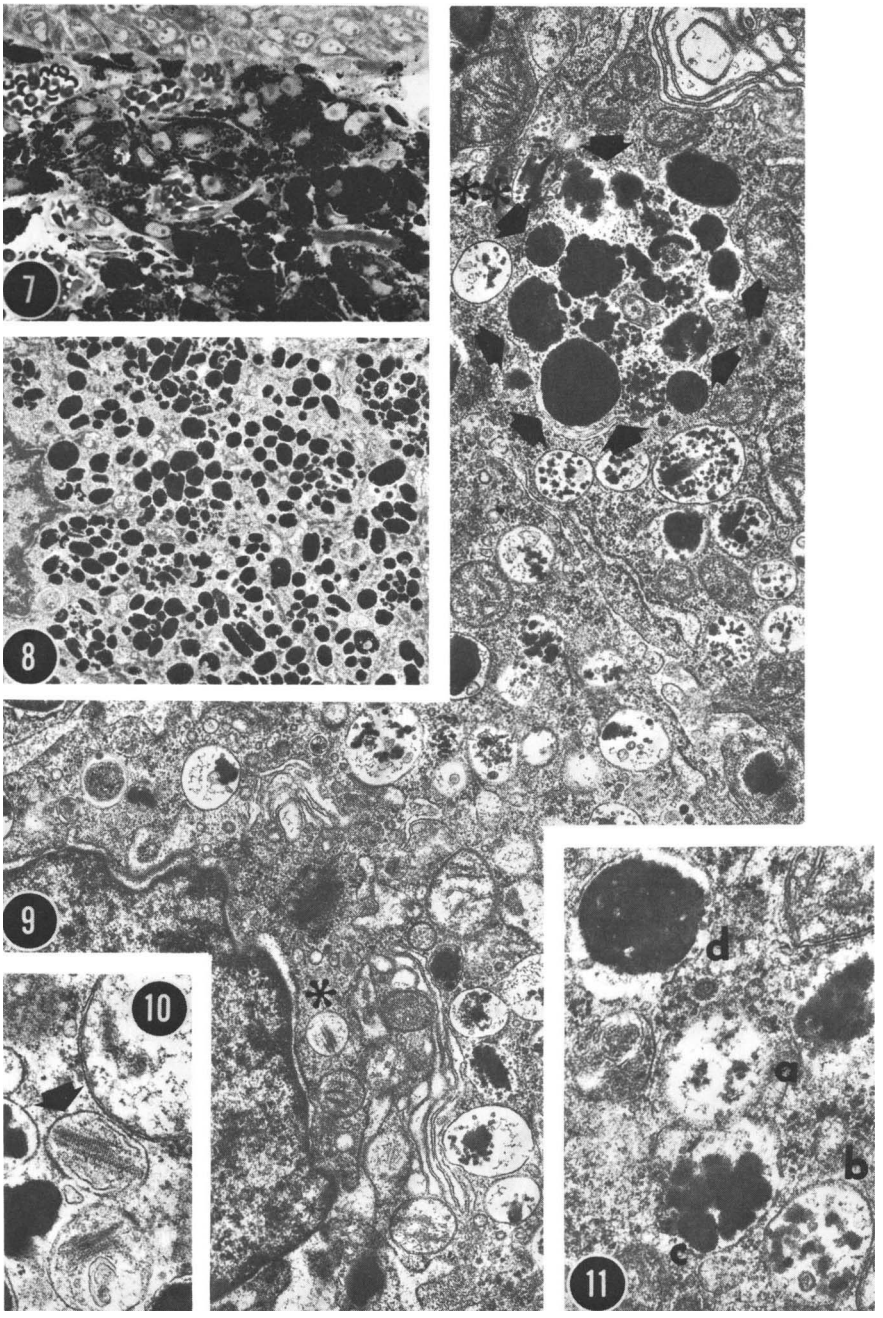

FIG. 7. Light micrograph of dermal melanotic tumor (×339). (Orientation for electron micrographs of Figs. 8–11).

FIG. 8. Portion of a tumor cell containing numerous fully melanized round-elliptical (Stage IV) melanosomes (×5580).

FIG. 9. Portion of tumor cell illustrating melanosome synthesis and autophagocytosis (×14,570). *Stage II melanosome with parallel arrays of filaments (see Fig. 10); **"compound melanosomes" (autophagosome) surrounded by a membrane which appears to be discontinuous (arrows). The remaining round melanosomes illustrate various stages in melanosomal development via melanization and aggregation of disorganized vesicular and fibrillar elements (see Fig. 11).

pigmentation and blue nevus-like tumors induced in the dorsal trunk skin of Mongolian gerbils by DMBA. Hyperpigmented dermis was also examined in gerbils treated with DMBA and croton oil.

The dermal hyperpigmentation was characterized by numerous melanin-bearing melanocytes and macrophages. Some of the dermal melanocytes were loaded with fully melanized (Stage IV) melanosomes, whereas others contained both mature melanosomes and immature ones (Stages I–III). Some dermal melanocytes displayed giant melanosomes ("macromelanosomes") along with melanosomes of normal size. Finally, occasional dermal melanocytes exhibited typical Stages I–III melanosomes as well as round Stage I-like melanosomes in which melanin deposition occurred prematurely on the vesicular and filamentous matrices. The macrophages of hyperpigmented dermis contained groups of melanosomes segregated within membrane-limited secondary lysosomes.

Paralleling the diversity in melanosome formation within the dermis, examination of DMBA-induced blue nevus-like tumors indicated that melanosomes were formed (1) in the standard manner with melanin deposited on a matrix of aligned filaments within a membrane-limited vacuole, and (2) by melanin deposition on a disorganized matrix of nonaligned filaments and/or microvesicular bodies within a membrane-limited vacuole. Melanosomes formed via both pathways were subsequently grouped within autophagosomes and degraded therein.

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FIG. 10. Two early melanosomes within a melanotic tumor cell. The arrow indicates a typical Stage II melanosome which lies just above a presumed Stage I melanosome. The latter exhibits a disorganized accumulation of lamellar, vesicular, and myelin-like bodies (×29,450).

FIG. 11. Melanosome synthesis via melanization and aggregation of vesicular and fibrillar elements within round granules; (a–d) indicates a possible developmental sequence (×29,450). A similar process of melanization and subsequent maturation of melanosomes within dermal melanocytes is shown in Fig. 5.

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