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Melanosomes Are Transferred from Melanocytes to Keratinocytes through the Processes of Packaging, Release, Uptake, and Dispersion

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Recent studies have described the role of shedding vesicles as physiological conveyers of intracellular components between neighboring cells. Here we report that melanosomes are one example of shedding vesicle cargo, but are processed by a previously unreported mechanism. Pigment globules were observed to be connected to the filopodia of melanocyte dendrites, which have previously been shown to be conduits for melanosomes. Pigment globules containing multiple melanosomes were released from various areas of the dendrites of normal human melanocytes derived from darkly pigmented skin. The globules were then captured by the microvilli of normal human keratinocytes, also derived from darkly pigmented skin, which incorporated them in a protease-activated receptor-2 (PAR-2)-dependent manner. After the pigment globules were ingested by the keratinocytes, the membrane that surrounded each melanosome cluster was gradually degraded, and the individual melanosomes then spread into the cytosol and were distributed primarily in the perinuclear area of each keratinocyte. These results suggest a melanosome transfer pathway wherein melanosomes are transferred from melanocytes to keratinocytes via the shedding vesicle system. This packaging system generates pigment globules containing multiple melanosomes in a unique manner.

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INTRODUCTION

It is known that there are two major types of extracellular secretory vesicles that differ in many aspects, one type being exosomes and the other type being shedding vesicles. Exosomes are vesicles segregated within the intracellular multivesicular body lumen that are released into the extracellular space by exocytosis. In contrast, shedding vesicles are secreted vesicles that bud directly from the plasma membrane into the extracellular space. Recent studies have revealed that shedding vesicles participate in a variety of important biological processes such as the surface-membrane traffic and the horizontal transfer of proteins and RNAs among neighboring cells; however, knowledge of shedding vesicles is still limited (Cocucci *et al.*, 2009).

Solar UV radiation is absorbed by DNA and damages nuclei in epidermal cells, which can lead to the formation of mutations. In the basal layer of the epidermis, there are specialized cells named melanocytes that produce melanin. One role of melanin is to prevent UV-induced nuclear DNA damage of human skin cells by screening out harmful UV radiation. Melanocytes produce specific organelles, termed melanosomes, in which melanin pigment is synthesized and deposited. In the skin, melanosomes are transferred from melanocytes to neighboring keratinocytes in order to form perinuclear melanin caps (Hearing, 2005).

A thorough understanding of melanosome transfer is crucial not only for protecting nuclear DNA from UV damage, but also for designing treatments for hyper- and hypo-pigmentary disorders of the skin, such as melasma, age spots, and vitiligo. Three possible mechanisms of melanosome transfer have been proposed: (1) direct inoculation of melanosomes into keratinocytes via keratinocyte–melanocyte membrane fusions through nanotubular filopodia, (2) release of individual melanosomes from melanocytes and their uptake by keratinocytes via phagocytosis, and/or (3) partial cytophagocytosis of melanocyte dendrite tips containing melanosomes by adjacent keratinocytes (Mottaz and Zelickson, 1967; Yamamoto and Bhawan, 1994; Seiberg, 2001; Scott *et al.*, 2002; Van Den Bossche *et al.*, 2006; Singh *et al.*, 2008). The finding that protease-activated receptor-2 (PAR-2), a seven-transmembrane G-protein-coupled receptor expressed by keratinocytes (Seiberg *et al.*, 2000a), regulates

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Abbreviations: D-PBS, Ca^{2+} and Mg^{2+}-free Dulbecco's phosphate-buffered saline; PAR-2, protease-activated receptor-2; PMA, phorbol 12-myristate 13-acetate; SEM, scanning electron microscopy; STI, soybean trypsin inhibitor; TEM, transmission electron microscopy

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melanosome transfer via keratinocyte phagocytosis (Sharlow *et al.*, 2000; Seiberg *et al.*, 2000a, b; Boissy, 2003; Cardinali *et al.*, 2005) reinforces the notion that the release of melanosomes from melanocytes, at least in part, is involved in the machinery of melanosome transfer. Furthermore, melanosome transfer is known to be skin-color dependent, and it is the racial and ethnic origin of the keratinocytes that determine the distribution patterns of transferred melanosomes in the recipient keratinocytes (Minwalla *et al.*, 2001; Thong *et al.*, 2003; Yoshida *et al.*, 2007). However, the exact mechanism of the transfer process of melanosomes from melanocytes to keratinocytes has not been fully clarified.

Recently, a new mechanism for the transfer of melanosomes was reported in which pigment globules containing multiple melanosomes are released into the extracellular space from melanocytes and are then ingested by keratinocytes (Ando *et al.*, 2011). In this study, we expand knowledge about a new pathway of melanosome transfer, in which pigment globules are released into the extracellular space from various areas of melanocyte dendrites. The melanosomes incorporated within these pigment globules are then dispersed into the cytosol of keratinocytes within a few days following the gradual degradation of the membrane that surrounds each melanosome cluster. Finally, we show that this process has a role in the distribution of melanosomes around the perinuclear areas of keratinocytes.

RESULTS

Pigment globules containing multiple melanosomes are generated from various areas of melanocytes

Although the pigment globules observed in our previous study seemed to be generated from the tips of melanocyte dendrites (Ando *et al.*, 2011), the plasma membrane staining of normal human melanocytes derived from darkly pigmented skin in this study revealed that the pigment globules were generated from various areas of melanocyte dendrites (Figure 1a). Observation of the melanocyte surface by scanning electron microscopy (SEM) revealed multiple pigment globules adhering to the melanocyte dendrites (Figure 1b). At higher magnification, the pigment globules were observed to be covered with a membrane that appeared to be filled with melanosomes (Figure 1c). Some pigment globules being produced showed that the tips of nanotubular filopodia, conduits for melanosomes (Scott *et al.*, 2002; Singh *et al.*, 2010), were connected to the pigment globules as if they had deposited the melanosomes layer by layer upon the globules (Figure 1d). The observation of a pigment globule emerging from a melanocyte by transmission electron microscopy (TEM) revealed that multiple filopodia on the melanocyte were closely associated with the pigment globule (Figure 1e). At 24 hours after fluorescence staining of the plasma membranes of normal human melanocytes, pigment globules (diameter 2–7 μm) coated with the fluorescent plasma membrane were released into the culture medium and were collected and isolated using filtration through an 8-μm microporous membrane filter (Ando *et al.*, 2011) (Figure 1f). When the insides of these isolated pigment globules were observed by TEM, it was seen that multiple melanosomes were packed within them at a very high density (Figure 1g). When the

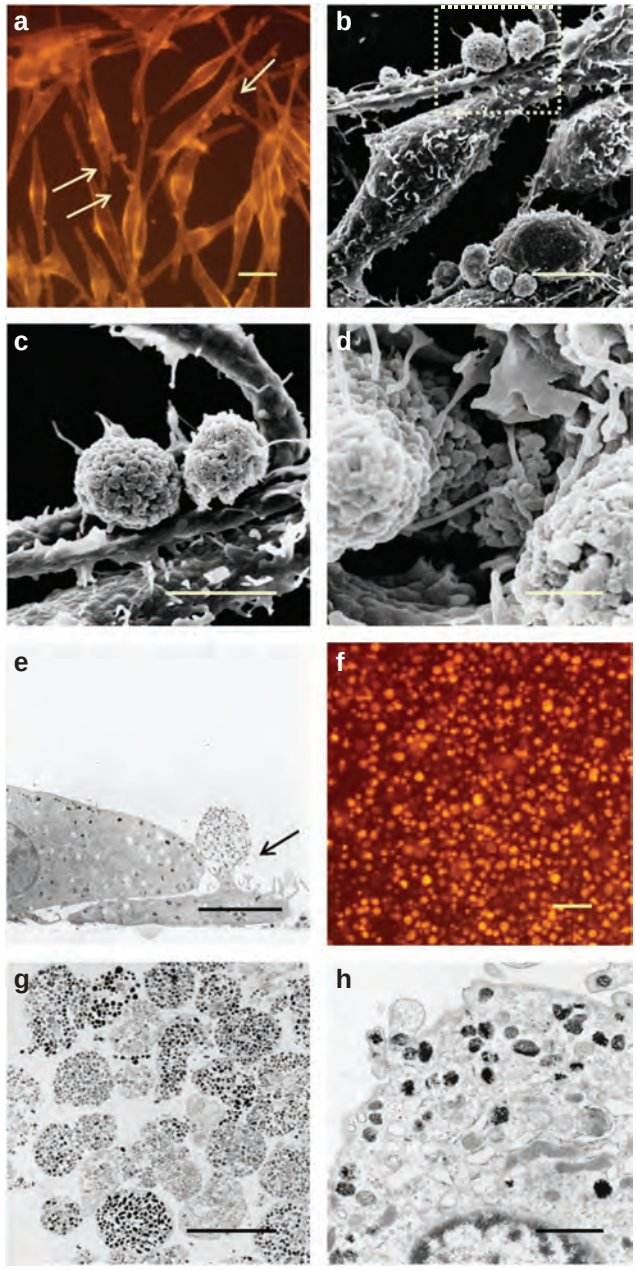

Figure 1. Pigment globules containing multiple melanosomes are generated from various areas of normal human melanocytes. (**a**) Fluorescence microscopic image of the plasma membrane staining of normal human melanocytes. Multiple globules (arrows) were generated from various areas on the melanocyte surface. Bar = 20 μm. (**b**) Scanning electron microscopy (SEM) image of normal human melanocytes in **a**. Bar = 5 μm. (**c**) Higher magnification image of the boxed region in **b**. Bar = 3 μm. (**d**) Pigment globules showing that the tips of melanocyte-derived nanotubular filopodia are connected to the globules. Bar = 1 μm. (**e**) Transmission electron microscopy (TEM) image of a pigment globule emerging from a melanocyte dendrite in a region of multiple filopodia (arrow). Bar = 5 μm. (**f**) Fluorescence microscopic image of globules isolated from the culture medium in **a**. Bar = 20 μm. (**g**) TEM image of the globules in **f**. Bar = 5 μm. (**h**) TEM image of a normal human melanocyte. Bar = 1 μm.

density of melanosomes in cytoplasmic areas of melanocytes was compared with the pigment globules, the density was much higher in the pigment globules than in the melanocyte cytoplasm (Figure 1h), indicating that the melanosomes are concentrated in the pigment globules.

Pigment globules are trapped by microvilli and are phagocytosed by keratinocytes in a PAR-2-dependent manner

To investigate the manner in which the pigment globules are incorporated into keratinocytes, light and electron microscopic observations were performed. At 24 hours after the addition of pigment globules to the culture medium of keratinocytes derived from darkly pigmented skin that were growing in the exponential phase, both sphere structures and flat structures of keratinocytes were observed with close association of the pigment globules (Figure 2a). SEM observation revealed that the sphere structure of keratinocytes generated multiple microvilli (diameter ~80 nm) from the surface, and they wrapped around and trapped the pigment globules (Figure 2b and c). The microvillus-associated trapping of the pigment globules was observed only by sphere-shaped keratinocytes but not by the flat keratinocytes. A later stage of keratinocyte phagocytosis that showed the wrapping stage of the pigment globules was also observed, where the pigment globules were engulfed by extensions of the keratinocyte plasma membrane (Figure 2d). TEM observation clearly showed the trapping of pigment globules juxtaposed with the keratinocyte membrane (Figure 2e) and they were finally covered with the membrane, resulting in their incorporation into the keratinocyte cytoplasm (Figure 2f). This structure is similar to the membrane-bound melanosome clusters observed in keratinocytes derived from darkly

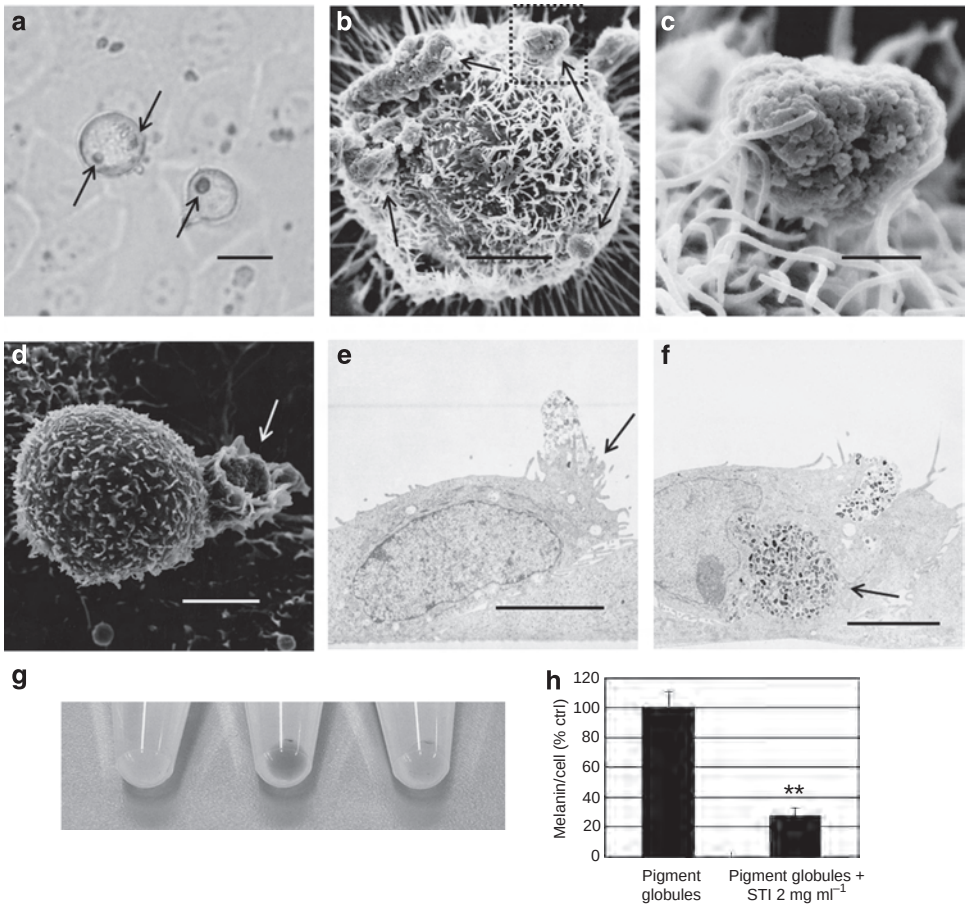

Figure 2. Pigment globules are trapped by microvilli and are phagocytosed by keratinocytes in a protease-activated receptor-2 (PAR-2)-dependent manner. (**a**) Light microscopic image of normal human keratinocytes after incubation with pigment globules for 24 hours. Pigment globules in sphere structures on keratinocytes (arrows) are observed. Bar = 20 μm. (**b**) Scanning electron microscopy (SEM) image of a normal human keratinocyte in **a**; multiple globules (arrows) are captured on the surface of the keratinocyte. Bar = 5 μm. (**c**) Higher magnification image of the boxed region in **b**. Bar = 1 μm. (**d**) SEM image of a normal human keratinocyte showing that the globule is wrapped by an extension of the keratinocyte plasma membrane (arrow). Bar = 5 μm. (**e**) Transmission electron microscopy (TEM) image of a normal human keratinocyte incubated with pigment globules for 48 hours. The pigment globule (arrow) is trapped and juxtaposed with the keratinocyte membrane. Bar = 5 μm. (**f**) TEM image of a normal human keratinocyte incubated with pigment globules for 48 hours. A melanosome cluster (arrow) is incorporated in the keratinocyte. Bar = 5 μm. (**g**) Appearance of keratinocyte pellets after incubation with isolated pigment globules with (right) or without (middle) STI for 48 hours. The left pellet was not treated with pigment globules or STI. (**h**) Bar graphs of melanin content derived from melanosomes incorporated per keratinocyte after incubation with isolated pigment globules in the absence or presence of STI. Data are expressed as a percentage of control cells (pigment globules) untreated with STI (100%) and are mean values ± SD of triplicate determinations (**$P<0.01$ vs. the control). STI, soybean trypsin inhibitor.

pigmented skin cocultured with melanocytes (Minwalla *et al.*, 2001). Furthermore, the uptake of pigment globules could be inhibited by treatment with soybean trypsin inhibitor (STI), a known inhibitor of PAR-2 that is involved in the phagocytosis by keratinocytes. Thus, the pigmentation of cell pellets of keratinocytes incubated with the pigment globules and 2 mg ml^{-1} STI for 48 hours was visibly lighter than the pigment globule–incubated control in the absence of STI (Figure 2g). This indicates that the incorporation of pigment globules by normal human keratinocytes is PAR-2 dependent, in a manner similar to the internalization of latex microsphere beads (Cardinali *et al.*, 2005) or individual isolated melanosomes (Ando *et al.*, 2010). When the incorporated pigment globules were measured in the cell pellets shown in Figure 2g, the absorbance at 490 nm, which reflects the level of melanin, was decreased by 72% in the STI-treated keratinocytes compared with the untreated control (Figure 2h).

The membranes surrounding the incorporated melanosome clusters are gradually degraded and the melanosomes are dispersed around the perinuclear area of keratinocytes

Melanosomes incorporated into keratinocytes in human skin are usually observed to be dispersed around the perinuclear area and they form a sunscreen against solar UV radiation, termed supranuclear melanin caps (Byers *et al.*, 2003). The pigment globules incorporated into keratinocytes after 48 hours of incubation were composed of melanosome clusters, and therefore the time-dependent alteration of the structure of pigment globules after incorporation into keratinocytes was evaluated. Melanin pigments were identified by Fontana–Masson staining, and no staining was observed immediately after the addition of the pigment globules to the keratinocytes (Figure 3a). However, at 1 or 2 days after the addition of pigment globules to the keratinocytes, the shapes of the pigment globules incorporated in

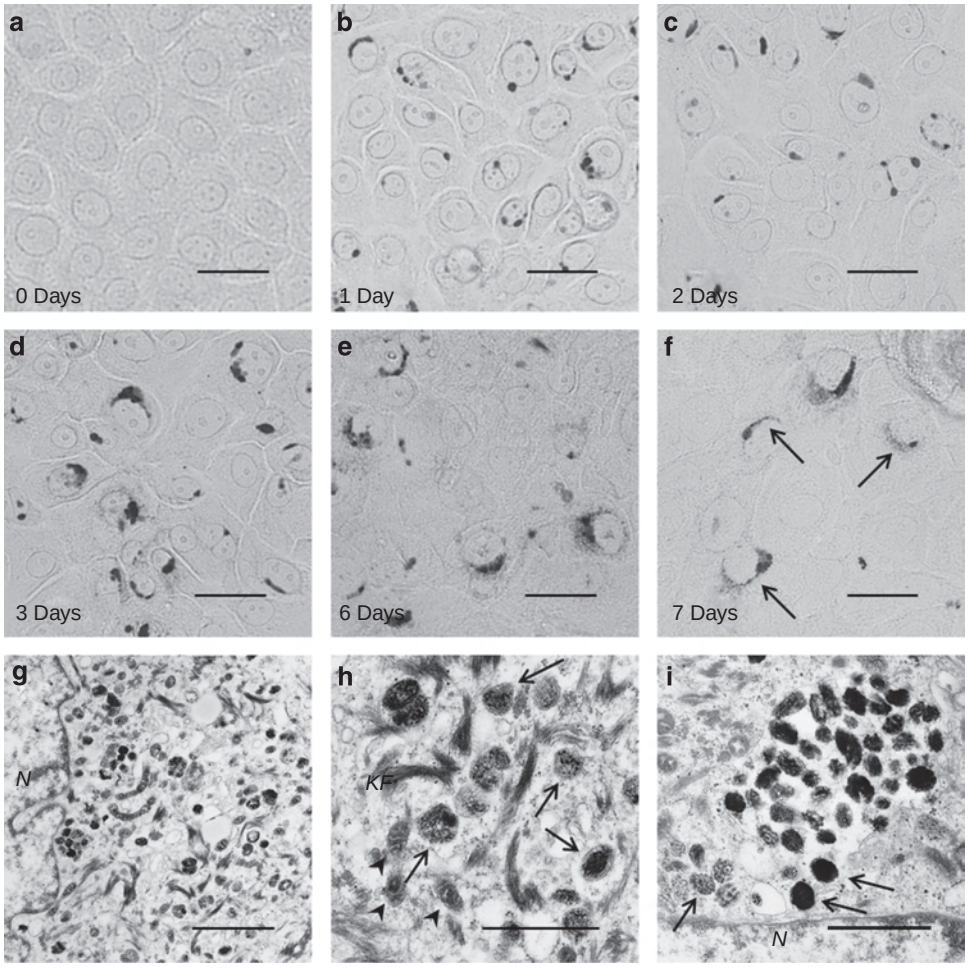

Figure 3. Membranes surrounding incorporated melanosome clusters in normal human keratinocytes are gradually degraded and the melanosomes are dispersed around the perinuclear area. (**a–f**) Light microscopic images of normal human keratinocytes (**a**) immediately after or after incubation with pigment globules for (**b**) 1 day, (**c**) 2 days, (**d**) 3 days, (**e**) 6 days, and (**f**) 7 days. All panels were stained with Fontana–Masson to detect melanin in the melanosomes. The fine staining appeared to be exuded from the incorporated pigment globules (arrows) that resulted in dispersion and uneven accumulation around the perinuclear area in **e** and **f**. In **a–f**, bars = 30 μm. (**g**) Transmission electron microscopy (TEM) image of a normal human keratinocyte in **e**. Bar = 2 μm. *N*, nuclei. (**h**) Higher magnification image of **g** showing that individual melanosomes (arrows) are dispersed among the keratin fibers, together with mitochondria (arrowheads). Bar = 1 μm. *KF*, keratin fiber. (**i**) TEM image of a normal human keratinocyte showing that individual melanosomes are released from the cleavage of the membrane enclosure into the cytosol. Bar = 1 μm. *N*, nuclei.

keratinocytes were globular and were in the perinuclear area (Figure 3b and c). After 3 days, individual melanosomes were observed to be exuded from some of the incorporated pigment globules (Figure 3d). At 6 and 7 days, almost all the pigment globules incorporated in the keratinocytes disappeared as such, and individual melanosomes were dispersed and had accumulated unevenly around the perinuclear area (Figure 3e and f). When keratinocytes incubated with the pigment globules were observed by TEM, individual melanosomes without a surrounding membrane appeared in the cytosol of keratinocytes after 6 days of incubation with the pigment globules (Figure 3g). In contrast, the pigment globules incorporated after 2 days of incubation with the pigment globules showed an apparent membrane enclosure surrounding the melanosome clusters (Figure 2f). At higher magnification, individual melanosomes were observed to be dispersed among the keratin fibers that are produced only in keratinocytes but not in melanocytes (Figure 3h). In keratinocytes incubated with the pigment globules for 6 days, we observed that melanosomes were released into the cytosol following the cleavage of the degraded membrane that surrounded each melanosome cluster (Figure 3i).

DISCUSSION

Recent studies have shed light on vesicular carriers of the plasma membrane and cytosolic components into the extracellular space, such as exosomes and shedding microvesicles. Exosomes are vesicles with diameters of 40–100 nm contained in multivesicular bodies that are secreted upon fusion of multivesicular bodies with the cell surface plasma membrane (Simons and Raposo, 2009). They not only carry proteins and lipids but also nucleic acids between different cells for intercellular communication (Valadi *et al.*, 2007; Simpson *et al.*, 2008). Examples of melanogenic proteins that are sorted in exosomes are Pmel17/gp100, 5,6-dihydroxyindole-2-carboxylic acid oxidase (tyrosinase-related protein 1), and MART-1 (melanoma antigen recognized by T cells) (Andre *et al.*, 2002; Theos *et al.*, 2006). As melanosomes in normal human melanocytes are distributed individually in the cytosol with no surrounding membranes (Figure 1h), this indicates that melanosomes themselves are not a type of exosome enclosed by intracellular multivesicular bodies. In contrast, shedding microvesicles are vesicles thought to be larger than exosomes with diameters up to 1 μm that bud directly from the plasma membrane into the extracellular space (Cocucci *et al.*, 2009). Shedding vesicles are surrounded by a plasma membrane, whereas exosomes are not. Although the diameter of the pigment globules observed in this study was larger than shedding vesicles (i.e., 1 μm vs. 2–7 μm), the pigment globules have characteristics that can be categorized into a kind of shedding vesicle because they are released into the extracellular space enclosed by a plasma membrane and they transfer intracellular organelles and components to neighboring cells in a manner distinct from exocytosis. In addition, our previous study showed that pigment globules are not apoptotic bodies that contain DNA fragments, as no nuclear staining of 4,6-diamidino-2-phenylindole is observed in the isolated pigment globules (Ando *et al.*, 2011).

The plasma membrane staining of this study allowed the outline of pigment globules to be distinguished, and it permitted the fluorescence microscopic observation of pigment globules attached to various areas of melanocyte dendrites. To transfer multiple melanosomes from melanocytes to keratinocytes promptly and efficiently, it seems reasonable that the pigment globules are generated not only from the tips but also from various areas of the dendrites of normal human melanocytes. The chances of pigment globules encountering recipient neighboring keratinocytes are much higher from all areas of the dendrites rather than only from the tips. In addition, the mechanism of transferring melanosomes within packages is also more prompt and efficient in delivering large numbers of them at a time as opposed to transferring individual melanosomes separately. Taking these observations into account, our findings make it seem likely that the delivery system described here is functional in human skin. The pigment globules detach from the melanocyte dendrites and are released into the culture medium under the present experimental conditions. However, as melanocytes and keratinocytes are closely adjacent to each other in the epidermis, the ingestion of not-yet-shed pigment globules attached to melanocyte dendrites by keratinocytes cannot be ruled out (Figure 4).

The release of melanosomes from melanocytes has been observed in previous studies, e.g., the appearance of membrane vesicles that contain small numbers of melanosomes from human melanoma cells (Cerdan *et al.*, 1992), the chemical analysis of melanin released into the culture medium from murine melanocytes (Virador *et al.*, 2002), and, more recently, the release of pigment globules containing multiple melanosomes into the culture medium by normal human melanocytes (Ando *et al.*, 2011). In this study, it was observed that multiple filopodia are connected to the pigment globules under construction (Figure 1d). Although knowledge of the mechanism used to generate shedding vesicles from the plasma membrane is still limited, the construction of pigment globules wrapped by the plasma

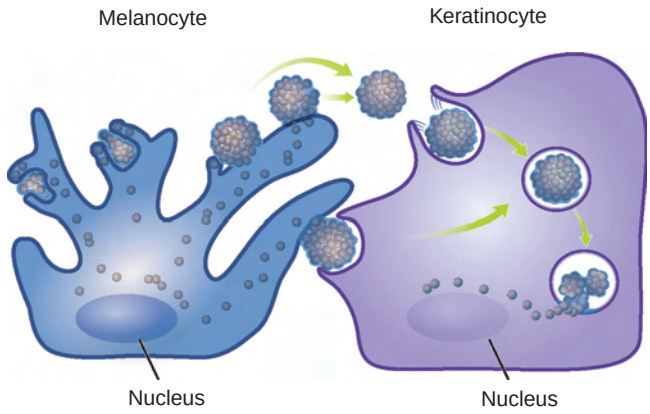

Figure 4. Scheme depicting the melanosome transfer pathway. Melanosomes are packed in globules enclosed by the melanocyte plasma membrane, released into the extracellular space from various areas of the melanocyte dendrites, phagocytosed by keratinocytes, and are then dispersed around the perinuclear area.

membrane seems to be a unique process. Filopodia generated from the melanocyte dendrites were originally found to serve as conduits for melanosome transfer to keratinocytes (Scott *et al.*, 2002). Recently, it was also found that the ''filopodial-phagocytosis model'' is involved in melanosome transfer, in which melanosome-containing filopodia are inserted into keratinocytes, which results in their phagocytosis (Singh *et al.*, 2010). Here we show that filopodia are closely related to the production of pigment globules; however, a distinct point of this study is that once filopodia detach from the globules, melanosomes in the pigment globules are released and incorporated by microvillus-associated phagocytosis into keratinocytes. This mechanism is distinct from previous studies that showed that melanosomes are directly inserted into keratinocytes through the filopodia conduits.

One of the interesting findings of this study is that pigment globules are degraded gradually after they are incorporated into keratinocytes and the multiple melanosomes within the pigment globules are dispersed into the cytosol and accumulate in the perinuclear area. The light microscopic observation by Fontana–Masson staining clearly shows that the distribution pattern of melanosomes changes in a time-dependent manner (Figure 3b and c vs. 3e and f). The TEM observations also reveal that the dispersion of melanosomes from the pigment globules in the keratinocyte cytosol is initiated by the destruction of the membrane surrounding the melanosome cluster (Figure 3i). It is well known that keratinocytes have a pivotal role in regulating the distribution patterns of recipient melanosomes. Melanosomes in lightly pigmented skin–derived keratinocytes are often distributed in membrane-bound clusters of melanosomes, whereas melanosomes in darkly pigmented skin–derived keratinocytes are predominantly individually dispersed (Minwalla *et al.*, 2001; Thong *et al.*, 2003). The human skin substitute model composed of cells derived from different skin pigmentation types clearly demonstrated that keratinocytes have a significant role in skin color by regulating melanogenesis and determining the eventual distribution of transferred melanosomes (Yoshida *et al.*, 2007). In this study, the pigment globules incorporated in darkly pigmented skin–derived keratinocytes were found to be in membrane-bound clusters of melanosomes within a few days and then dispersed individually later. Therefore, it is possible that the degradation of pigment globules or the dispersal of melanosome clusters might remain incomplete, which results in static clusters of melanosomes, in lightly pigmented skin–derived keratinocytes. Thus, the distribution patterns of melanosomes could be different if keratinocytes derived from lightly pigmented skin had been used in this study. In addition, it was recently reported that the loss of fluorescently labeled and isolated melanosomes, possibly due to the degradation of melanosomes themselves, was observed after incorporation in cultured human keratinocytes (Ebanks *et al.*, 2011). Although the electron density of free melanosomes in the keratinocyte cytosol is apparently lower than that of the packed melanosomes within the membrane enclosure (Figure 3h and i), the pathway for the degradation of melanosomes after their dispersion in the keratinocyte cytosol remains to be further elucidated.

In conclusion, our observations in this study strongly suggest a previously unreported mechanism of melanosome transfer wherein melanosomes are concentrated in pigment globules via the filopodia. These globules then bud off from various areas of melanocyte dendrites, are released into the extracellular space, are phagocytosed by keratinocytes, and are then distributed individually around the perinuclear area, followed by gradual degradation of the surrounding membrane. However, whether a single transfer mechanism or multiple mechanisms are involved in the melanosome transfer pathway still remains unclear. The experimental systems for studying melanosome transfer presented in this study may be useful tools to not only investigate the mechanism(s) of intercellular logistics via the shedding microvesicle systems, but also design new concepts for treating hyper- and/or hypo-pigmentary disorders of the skin based on additional melanosome transfer machineries.

MATERIALS AND METHODS

Cell culture

Cultures of normal human epidermal melanocytes (derived from darkly pigmented newborn foreskins, passage up to 4; Cascade Biologics, Portland, OR) were maintained in medium 254 (Cascade Biologics) supplemented with a commercial cocktail of growth factors (HMGS, consisting of 10 ng ml^{-1} phorbol 12-myristate 13-acetate (PMA), 3 ng ml^{-1} human recombinant basic fibroblast growth factor, 3 μg ml^{-1} heparin, 500 nM hydrocortisone, 5 μg ml^{-1} insulin, 5 μg ml^{-1} transferrin, 0.2% (v/v) bovine pituitary extract, and 0.5% (v/v) fetal bovine serum) and an antibiotic/antimycotic solution (A5955; Sigma-Aldrich, St Louis, MO) at 37 °C in a humidified atmosphere with 5% CO_2. Normal human epidermal keratinocytes (derived from darkly pigmented newborn foreskins, passage 3; Cascade Biologics) were cultured in medium 154 (Cascade Biologics, 0.2 mM Ca^{2+}) supplemented with a commercial cocktail of growth factors (HKGS, consisting of 0.2 ng ml^{-1} human recombinant EGF, 0.18 μg ml^{-1} hydrocortisone, 5 μg ml^{-1} insulin, 5 μg ml^{-1} transferrin, and 0.2% (v/v) bovine pituitary extract) and an antibiotic/antimycotic solution.

Plasma membrane staining of melanocytes

The plasma membrane staining of normal human melanocytes was performed by incubation with 5 μg ml^{-1} CellMask Orange (Invitrogen, Molecular Probes, Carlsbad, CA) for 3 minutes. After washing the cells with melanocyte culture medium three times, the cells were observed by fluorescence microscopy ($\lambda_{ex} = 554$ nm, $\lambda_{em} = 567$ nm).

Isolation of pigment globules from the melanocyte culture medium

Normal human melanocytes were seeded at 2×10^6 cells per 10 ml medium 254 supplemented with a commercial cocktail of growth factors (HMGS) and an antibiotic/antimycotic solution in 10-mm cell culture dishes. After 24 hours, the culture medium was collected. The collected culture media were centrifuged at $2 \times 10^3 g$ for 10 minutes at room temperature to precipitate pigment globules and floating melanocytes. After the supernatants were decanted, the pellets were resuspended in 1 ml of medium 254 supplemented with HMGS-2 (consisting of 10 nM endothelin-1 instead of PMA) to remove PMA before the pigment globules were added to

keratinocytes, as PMA causes differentiation of keratinocytes (Dlugosz and Yuspa, 1993; Matsui *et al.*, 1993). Each suspension was then further centrifuged at 2×10^4 *g* for 5 minutes at room temperature and the supernatant was decanted. Each pellet was sequentially suspended by pipetting 20 times in HMGS-2-containing medium 254 in order to dissect out pigment globules and melanocytes. The suspension was then poured into a hanging cell culture insert, six-well Millicell 8 μm PET (PIEP30R48; Millipore, Billerica, MA), touching the bottom of a 60-mm cell culture dish, and the culture medium that penetrated the filter to remove floating melanocytes was collected slowly by capillary action. The collected medium was finally centrifuged at 2×10^4 *g* for 5 minutes at room temperature and the pellet was used as the isolated pigment globule fraction (Ando *et al.*, 2011). The pellets were covered with a small amount of culture medium to prevent desiccation and were stored at −20 °C until use.

Incubation of normal human keratinocytes with pigment globules in the absence or presence of STI

Pellets consisting of isolated pigment globules obtained from four of the 100 mm cell culture dishes were resuspended in 100 μl medium 254 supplemented with HMGS-2 using a pipette 20 times. The entire suspension of pigment globules was added to fresh culture medium in collagen I–coated six-well plates (35-4400; Becton Dickinson Labware, Bedford, MA) in which keratinocytes had been seeded at 5×10^5 cells per 2 ml per well 24 hours earlier. In culture dishes treated with STI, the culture medium was replaced with the same fresh medium containing 2 mg ml^{-1} STI in advance. After the indicated duration of incubation with pigment globules, keratinocytes adhering to the plates were gently washed three times with Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (D-PBS) to remove noningested pigment globules. Some dishes were used for SEM/TEM observation or Fontana–Masson staining. To the other dishes, 1 ml trypsin/EDTA solution (25300; Sigma-Aldrich) was added to each well for 10 minutes at 37 °C and the detached cells were harvested. Cell numbers were counted using a hemocytometer chamber to evaluate pigmentation. After centrifugation at 2×10^4 *g* for 5 minutes, each cell pellet was washed with D-PBS. After further centrifugation, the cells were used for TEM or to evaluate pigmentation.

Measurement of melanosome content in keratinocytes

To measure amounts of melanosomes incorporated into keratinocytes, the cell pellet obtained from each six-well plate was dispersed in 100 μl distilled water with careful mixing after which 400 μl ethanol/ether 1:1 (v/v) was added to remove opaque substances other than melanin (Ando *et al.*, 1999). This mixture was briefly suspended and stored at room temperature for 5 minutes. After successive centrifugation at 1×10^3 *g* and at 1.5×10^4 *g* for 5 minutes each, the precipitates were solubilized with 110 μl 10% DMSO in a 1 M NaOH aqueous solution at 80 °C for 10 minutes in a safe-lock Eppendorf tube (1.5 ml) with brief mixing every 5 minutes. After a brief low-speed centrifugation using a tabletop centrifuge, 100 μl of each mixed solution was transferred to a 96-well plate, and the absorbance at 490 nm was used to measure melanin concentration. The background absorbance of keratinocytes at 490 nm (with no incubation with pigment globules and STI) was subtracted from the values of cells incubated with pigment globules in the absence or presence of STI. Melanin content per cell was calculated and is expressed as a percentage of control cells.

Light microscopic observation of melanosomes incorporated into keratinocytes

For light microscopic observations of incorporated melanosomes, Fontana–Masson staining was performed. Normal human keratinocytes grown in collagen I–coated 35 mm cell culture dishes with or without incubation with pigment globules were washed twice with D-PBS, and then were fixed with cold methanol for 10 minutes at 4 °C, washed twice with distilled water, incubated with the Fontana ammoniacal silver solution (Muto Pure Chemicals, Tokyo, Japan) for 1 hour at 37 °C in a thermostatic oven, and finally washed twice with distilled water (Ando *et al.*, 2010).

Electron microscopy

To observe the cell surface of cultured normal human melanocytes and keratinocytes by SEM, the cells grown on poly-D-lysine-coated 35-mm glass-bottom culture dishes (MatTek, Ashland, MA) were fixed with 2% glutaraldehyde in the keratinocyte culture medium at 4 °C for ⩾2 hours. After washing twice with D-PBS for 15 minutes each, the cells were postfixed with 2% osmium tetroxide for 1.5 hours. After fixation, they were dehydrated in a graded series of ethanol. These dehydrated cells were covered with t-butyl-alcohol, freeze-dried (JFD-310; JEOL, Tokyo, Japan), coated with a layer of sublimated OsO_4 using an osmium plasma coater (OPC80, Filgen, Nagoya, Japan), and examined by SEM (JSM3620F; JEOL) at 5 kV. To observe intracellular organelles of normal human melanocytes and keratinocytes by TEM, cells incubated with the pigment globules were washed with D-PBS or harvested with trypsin/EDTA, and fixed with 2% glutaraldehyde in D-PBS at 4 °C for ⩾2 hours. For the observation of pigment globules, the isolated pigment globules were fixed with 2% glutaraldehyde in the keratinocyte culture medium at 4 °C for at least 2 hours. After washing twice with D-PBS for 15 minutes each, the cells or the pigment globules were postfixed with 2% osmium tetroxide for 1.5 hours. After fixation, they were dehydrated in a graded series of ethanol, and embedded in epoxy resin, Quetol812 (Nisshin EM, Tokyo, Japan) for 48 hours at 60 °C. Ultrathin sections were cut and then stained with uranyl acetate and lead citrate, and were examined using an electron microscope (JEM-1200EX; JEOL) at 80 kV.

Statistical analysis

Statistical analysis was performed on a minimum of three independent experiments using one-way analysis of the Dunnet II test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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