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Disruption of melanosome transport in melanocytes treated with theophylline causes their degradation by autophagy

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ABSTRACT

Melanosomes containing melanin are transported from the perinuclear area to the tips of dendrites in epidermal melanocytes, and are then transferred to keratinocytes. Thus, skin color is determined by the amount of melanin synthesized in melanocytes and the subsequent dispersion of melanosomes in the epidermis. Therefore, disrupting intracellular melanosome transport in melanocytes is considered an effective approach to regulate skin color. However, the fate of melanosomes that accumulate in melanocytes due to disrupted intracellular transport is unclear. In this study, we disrupted melanosome transport by knockdown of the motor protein MyosinVa. Knock-down of MyosinVa (M-KD) in cells treated with theophylline significantly down-regulated the mRNA and protein expression levels of tyrosinase. Interestingly, intracellular melanin contents in M-KD cells were decreased. Furthermore, M-KD cells showed activation of autophagy through increased expression of Microtubule-associated protein 1 light chain 3 (LC3) -II and decreased expression of p62. The sum of these results indicate that disruption of melanosome transport causes their degradation by autophagy.

1. Introduction

Melanin plays critical roles in protecting human skin against genotoxic damage from the UV radiation in solar light and in maintaining skin homeostasis [1]. However, excess melanin production, namely hyperpigmentation, often is associated with skin cancer. In addition, localized hyperpigmentation of the skin usually influences the appearance of individuals as a sign of aging [1,2].

Melanin is synthesized by tyrosinase, which is the rate-limiting enzyme, and by tyrosinase-related proteins-1 (TRP-1) and -2 (TRP-2) in melanosomes, which are lysosome-related organelles [3,4]. Excessive melanin synthesis is caused by the activation of melanocytes. Since melanocytes are surrounded by keratinocytes in the skin, melanin synthesis is strongly affected by keratinocytes, and several signaling pathways involved via keratinocytes have already been identified. For instance, the proliferation of melanocytes and tyrosinase synthesis in melanocytes are stimulated by several secreted factors, such as interleukin-1α (IL-1α), endothelin-1 (ET-1), stem cell factor (SCF) and α-melanocyte stimulating hormone (α-MSH), all of which are produced by keratinocytes following UV irradiation or environmental stress [5–7]. The melanin synthesized is exfoliated from the skin by the following process: First, melanosomes with accumulated melanin are intracellularly transported from the perinuclear area to the tips of dendrites of melanocytes. During that process, several motor proteins, such as Kinesin and MyosinVa, move melanosomes using microtubules and actin filaments [8–10]. Finally, melanosomes are incorporated into surrounding keratinocytes via globules and are then distributed throughout the skin [11].

To prevent hyperpigmentation of the skin, which can dramatically influence the appearance of individuals, approaches targeting tyrosinase have been commonly accepted. However, disrupting the intracellular transport or intercellular transfer of melanosomes with accumulated melanin has recently been discussed as an approach to avoid the risk of hypopigmentation, because vitiligo can occur following the application of whitening agents [12,13]. Although it has been reported that melanosomes intercellularly transferred to keratinocytes are degraded by autophagy [14], the fate of melanosomes that accumulate in melanocytes due to disrupted intracellular transport is still unknown.

Thus, this study was conducted to clarify the fate of melanosomes in melanocytes where intracellular transport was disrupted by the knockdown of MyosinVa in B16 mouse melanoma cells (M-

Abbreviations: LC3, Microtubule-associated protein 1 light chain 3; siMyosinVa, MyosinVa-small interfering RNA.

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KD cells). M-KD cells showed a reduction of intracellular melanin content compared with control cells in the presence of theophylline, which is an activator of cyclic AMP signaling [15]. Disrupting intracellular melanosome transport in M-KD cells caused the down-regulation of tyrosinase expression. Furthermore, those melanosomes disappeared from M-KD cells due to autophagy. Interestingly, these phenomena were only found in M-KD cells cultured in the presence of theophylline.

The study is the first report to suggest the fate and impact of melanosomes in melanocytes where intracellular transport was disrupted.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) and synthetic melanin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Nichirei Bioscience (Tokyo, Japan). Theophylline was purchased from Wako Pure Chemicals (Osaka, Japan). *Power* SYBR Green Cells-to-CT kit, SYBR Green Master Mix, PCR primers, MyosinVa-small interfering RNA (siMyosinVa), non-targeting siRNA (siControl) and Lipofectamine 2000 were purchased from Life Technologies (Carlsbad, CA, USA). RIPA buffer and protease inhibitor were purchased from Nacalai tesque (Kyoto, Japan). The BCA protein assay kit was purchased from Thermo (Waltham, CA, USA). Rabbit anti-LC3 and rabbit anti-p62 polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Donkey anti-rabbit IgG H&L (Alexa Fluor 555) and donkey anti-goat IgG H&L (Alexa Fluor 488) were purchased from Abcam (Cambridge, UK). The rabbit anti-tyrosinase and goat anti-Pmel17 polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) was purchased from Dojindo Laboratories (Kumamoto, Japan).

2.2. Cell culture

B16 mouse melanoma cells (B16 cells) were obtained from the Riken Cell Bank (Ibaraki, Japan), and were cultured in DMEM with 10% FBS at 37 °C in a 5% CO_2 atmosphere.

2.3. RNA interference

B16 cells were seeded into multi-well plates in DMEM, and were treated with 100 nM MyosinVa-small interfering RNA (siMyosinVa) or non-targeting siRNA (siControl) using Lipofectamine 2000 for 24 h. The cells were incubated with or without theophylline (1 mM) for 48 h, and then were used for the following examinations.

2.4. Intracellular melanin measurement

Melanin contents in cells were measured according to the protocol described previously [16].

2.5. Real-time PCR

Total RNAs were extracted from the cells and cDNAs were synthesized using a Power SYBR Green Cells-to-CT kit. Real-time PCR was performed with SYBR Green Master Mix using the StepOnePlus™ Real-Time PCR system (Life Technologies). The following primer sets for tyrosinase, MyosinVa and β-actin were used: tyrosinase forward 5′-CCTCCTGGCAGATCATTTGT-3′, tyrosinase reverse 5′-GGTTTT GGCTTTGTCATGGT-3′, MyosinVa forward 5′-AGTGCAGCAGCTAAGA GCAT-3′, MyosinVa reverse 5′-ATTCTTGCACGTTTGCTTTC-3′, β-actin forward 5′-GGGAAATCGTGCGTGACAT-3′, β-actin reverse 5′- CAG-GAGGAGCAATGATCTC-3’.

2.6. Western blotting

After the extraction of cellular proteins using RIPA buffer with protease inhibitors, the proteins were separated using 10% or 15% SDS-PAGE, and then were transferred to polyvinylidene difluoride membranes. The membranes were incubated with primary antibodies specific for tyrosinase (1:1000), LC3 (1:1000), p62 (1:1000) and β-actin (1:2000). Protein bands on the membranes were visualized using a WesternBreeze Chemiluminescent kit (Invitrogen, Carlsbad, CA, USA), and images were obtained using a ChemiDoc Touch Imaging System (Bio-Rad, Hercules, CA, USA).

2.7. Immunofluorescence

Cells were fixed with 4% formaldehyde, and then were permeabilized with 0.2% Triton X-100. After blocking nonspecific binding with 1% IgG-free BSA, 2 μg/mL rabbit anti-LC3 polyclonal antibody or goat anti-Pmel17 polyclonal antibody were used for immunofluorescent staining. Two μg/mL donkey anti-rabbit IgG H&L (Alexa Fluor 555) or donkey anti-goat IgG H&L (Alexa Fluor 488) were used to label antibodies that bound to LC3 and Pmel17. Cell nuclei were stained with DAPI. Confocal images were obtained using a BZ-X700 Fluorescence microscope (Keyence, Osaka, Japan).

2.8. Statistical analysis

All data are expressed as means ± SD. Comparisons between two groups were performed by Student's *t*-test. A *P*-value of less than 0.05 is considered statistically significant.

3. Results

3.1. Melanin content is decreased by the knockdown of MyosinVa

To examine the influence of MyosinVa knockdown on intracellular melanin content, we quantified melanin content in siMyosinVa-treated B16 mouse melanoma cells (M-KD cells). Although increased intracellular melanin content was expected due to the accumulation of melanosomes, the amount of intracellular melanin was significantly decreased in M-KD cells treated with theophylline, which is an activator of melanogenesis though cAMP signaling [15] (Fig. 1).

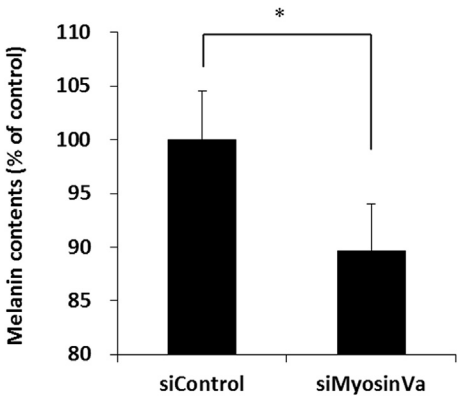

Fig. 1. Influence of MyosinVa knockdown on intracellular melanin content. M-KD cells were treated with theophylline (1 mM) for 48 h, and melanin contents in cells were measured. Bars indicate means ± S.D. (n = 3). *$p < 0.05$ indicates a significant difference between groups.