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Autophagy mediates anti-melanogenic activity of 3′-ODI in B16F1 melanoma cells

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ABSTRACT

Autophagy is a cellular degradation process for cellular aggregates and unneeded cellular compartments including damaged mitochondria, ER, and peroxisomes. Melanosome is cellular organelle that is the cellular site of generation, storage and transports of melanin in melanocytes. Despite potential importance of autophagy, the role of autophagy in melanogenesis and melanosome autophagy are largely unknown. In here, we identified 3′-hydroxydaidzein (3′-ODI) as an autophagy inducer from a phytochemical library screening. Treatment with 3′-ODI significantly reduced α-MSH-mediated melanogenesis but efficiently increased autophagy both in melanoma cells and melanocytes. Furthermore, inhibition of autophagy significantly reduced the anti-melanogenic effects of 3′-ODI in α-MSH-stimulated melanoma cells. Taken together, these results suggest that autophagy mediates anti-melanogenic activity of 3′-ODI.

1. Introduction

The color of the skin, hair, and eyes is derived from the biological pigmentation factor melanin, which is produced via melanogenesis within melanosomes that are specific cellular organelles in melanocytes [1]. Proper amount of melanin prevents the cellular damage induced by ultraviolet (UV) irradiation of the skin. However, hyper-pigmentation can be associated with a number of disease conditions. Melanins are produced in two different chemical forms, eumelanin and pheomelanin [2]. Several key players such as tyrosinase and tyrosinase-related protein 1/2 (TRP1/2) involved in melanin synthesis have been identified. These enzymes are involved in tyrosine metabolism, which generates melanin from tyrosine. Tyrosinase, which converts tyrosine to dopaquinone is responsible for the first rate-limiting step in tyrosine metabolism [3]. Indeed, mutations in tyrosinase result in the inherited pigmentary disorder albinism [4]. Thus, many tyrosinase inhibitors including arbutin and resveratrol have been used to regulate hyperpigmentation, and are used to treat skin pigmentation disorders. Microphthalmia-associated transcription factor (MITF) is the primary regulator for the expression of melanogenesis-related proteins [5,6]. MITF activity can be regulated at both the transcriptional and translational levels. The α-melanocyte-stimulating hormone (α-MSH) up-regulates MITF by transactivation of *MITF* via the cyclic adenosine monophosphate (cAMP)-signaling cascade. Additionally, phosphorylation of the MITF protein by ERK2 or p90/RSK6 regulates its degradation through the proteasome pathway [7]. Other transcriptional factors such as p53, HNF1-α, and Sox10 have also been suggested to be involved in the regulation of tyrosinase expression in melanocytes [2]. Since multiple genes and various cellular pathways are involved in the precise processes that contribute to melanogenesis are not yet fully defined.

Autophagy is a basic catabolic process and is responsible for degrading damaged or unwanted cellular components and organelles [8,9]. During autophagy, targeted substrates are isolated and engulfed in autophagosomes, which are then fused with lysosomes and finally degraded or recycled [10]. Autophagy-related genes (ATG) essentially control autophagosome formation and autophagy induction [11]. Autophagy ensures cell survival under stress conditions by supplying cellular energy sources. Nonetheless, autophagy is also associated with cell death processes [12]. Thus, autophagy plays important roles in homeostasis and in many pathophysiological conditions. Recent evidence has suggested that autophagy can participate in melanosome degradation and biogenesis [13]. Down-regulation of WIPI-1, a human homologue of the yeast ATG18 protein suppressed the transcriptional activation of

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MITF and its target genes. In addition, Murase et al. recently showed that autophagy affects skin color determination by regulation of melanin degradation in normal human epidermal keratinocytes [14]. However, despite its potential importance, the role of autophagy in melanogenesis is largely unknown.

In this study, we screened a phytochemical library and identified 3,7,4′-trihydroxyisoflavone (3-hydroxydaidzein, 3′-ODI), an *ortho*-dihydroxyisoflavone derivatives of fermented soybean paste as a potent autophagy inducer. Treatment with 3′-ODI efficiently inhibited melanogenesis but strongly activated autophagy both in B16F1 melanoma cells and Melan-a melanocytes. Furthermore, inhibition of autophagy significantly reduced the anti-melanogenic effects of 3′-ODI in melanocytes.

2. Materials and methods

2.1. Cell culture and stable cell line

The B16F1 mouse melanoma cell line was purchased from the American Type of Culture Collection (ATCC). And the Melan-a melanocytes was kindly provided by Dr. Dorothy C. Bennett (St. George's Hospital Medical School, London, UK). B16F1 cells were maintained at 37 °C in a 5% CO_2 humidified incubator and grown in Dulbecco's modified Eagle's medium (DMEM, Hyclone, ThermoScientific, Logan, UT) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Calsbad, CA), 1% penicillin/streptomycin (P/S). And Melan-a cells were cultured in RPMI 1640 medium (Hyclone) supplemented with 10% FBS, 1% P/S, and 200 nm phorbol-12-myristate-13-acetate (Sigma, St. Louis, MO). For GFP-LC3 stable expressing cells, B16F1 cells were transfected with pEGFP-LC3 using Lipofectamine according to manufacturer's protocol (Invitrogen, Carlsbad, CA). Stable transfectants (B16F1/GFP-LC3) were selected by growth in selection medium containing 1 mg/ml of G418 for 7 days. After single cell cloning, the stable clones were selected under a fluorescence microscope.

2.2. Reagents

3′-ODI is purified from Korean fermented soybean paste as previously reported [15]. α-MSH and Bafilomycin A1 were purchased from Sigma–Aldrich (St. Louis, MO). The expression plasmid pEGFP-LC3 was kindly provided by Dr. Noboru Mizushima (Tokyo Medical and Dental University, Japan) [16]. The previously validated siRNA for mouse ATG5 siRNA (5′-ACCGGAAACUCAUGGAAUA-3′) [17] and scrambled control siRNA (5′-CCUACGCCACCAAUUUCGU-3′) were synthesized from Bioneer (Daejeon, Korea).

2.3. Phytochemical library screening and autophagy analysis

For the image-based phytochemical library screening, B16F1/GFP-LC3 cells were seeded in a 96-well plate. Then, each chemical from the Library was treated to the each well with approximately 10 μM. Autophagy inducers were identified by monitoring the GFP-LC3 punctate structures under a fluorescence microscopy (IX71, Olympus, Japan). And autophagy activation was measured by counting of the number of cells with GFP-LC3 punctate structures under a fluorescence microscopy.

2.4. Melanin assay

The determination of melanin contents was performed using a slight modification of a previously described method [18]. Briefly, to measure the melanin contents, the cells were harvested by trypsinization and dissolved with soluble buffer (1 N NaOH containing 10% DMSO) at 100 °C for 30 min, and then, relative melanin content was determined by measuring at 415 nm using an ELISA plate reader (Victor X3, Perkinelmer).

2.5. Cell viability assay

B16F1 cells were seeded in 96-well plates. After treatment of 3′-ODI, the cells were incubated for 24 h. Cell viability was determined through the reduction of WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-*2H*-tetrazolium, monosodium salt] to water soluble formazan using a Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, kumamoto, Japan). CCK-8 was added to each well at 1/10 volume of media. The cells were incubated at 37 °C for 30 min. The absorbance change was measured at 450 nm using a micro plate reader.

2.6. Western blot analysis

The protein extract was isolated from cells using a 2× protein sample buffer (Bio-Rad). After separation in 10–15% SDS–PAGE, protein was transferred onto PVDF membrane and (Bio-Rad, Hercules, CA). The membrane was blocked with 5% skim milk in TBST for 1 h, and incubated with specific primary antibodies overnight at 4 °C. Anti-ATG5 (ab54033) antibody was purchased from Abcam (Cambridge, UK); anti-LC3 (NB100-2220) antibody was purchased from NOVUS Biologicals (Littleton, CO); anti-p62 (sc-28359) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Actin (MAB1501) antibody was purchased from Millipore (Temecula, CA); anti-tyrosinase and anti-TRP1 antibodies were kindly donated by V.J. Hearing (NIH, Bethesda, MD). For protein detection, the membranes were incubated with HRP-conjugated secondary antibodies and signals were detected with Super-signal West Dura HRP detection kit (Pierce, Rockford, IL).

2.7. Electron microscopy analysis

For transmission electron microscopy, cells treated with α-MSH (1 μM) for 24 h were incubated with 3′-ODI (100 μM) for another 1 day. Then the cells fixed in 4% glutaraldehyde (pH 7.4) containing 2% PFA in 30 mM phosphate buffer. After dehydration with ethanol, ultrathin sections were prepared using a Sorvall MT5000 microtome and collected on 150 mesh copper grids. Then, the sections were stained with 1% uranyl acetate and/or lead citrate. And images were obtained with a JEOL 100CX transmission electron microscope at 50 kV (JEOL, Japan).

2.8. Statistical analysis

The results were expressed as the means ± S.E.M. The probability of statistical differences between experimental groups was determined by the Student's *t*-test. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. 3′-ODI induces autophagy in B16F1 melanoma cells

Although autophagy eliminates cellular organelles, the possible degradation of melanosomes by autophagy has not been elucidated. To understand the role of autophagy in melanogenesis, we established a cell-based screening system in B16F1 mouse melanoma cells that stably expressing a molecular marker for autophagy, GFP-LC3 (B16F1/GFP-LC3). Using these cells, we screened a phytochemical library that consisted of anti-melanogenic agents. From the screening, we identified several candidates that induced autophagy in B16F1 melanoma cells. Among these candidates, we

selected 3′-ODI for further analysis as a potent autophagy inducer with anti-melanogenic activity (Fig. 1A). 3′-ODI is a major metabolite of the soy isoflanone, 4′,7-dihydroxyioflavone (diadzein) [19]. To confirm the screening results, B16F1/GFP-LC3 cells were exposed to different dose of 3′-ODI subsequently, autophagy activation was observed. Cells treated with 3′-ODI strongly increased LC3 protein conversion as well as the formation of GFP-LC3 punctuate structures, indicating that 3′-ODI efficiently induced autophagy in B16F1 melanoma cells (Fig. 1B–D). In addition, we also examined autophagic flux by 3′-ODI treatment. p62 protein, a substrate for autophagy, is used to measure autophagy flux. Indeed, p62 protein level was decreased in 3′-ODI -treated cells (Fig. 1D), further indicating that 3′-ODI efficiently induced autophagy in B16F1 melanoma cells. ATG5 is an essential player in autophagosome formation during autophagy. To examine the effect of ATG5 on 3′-ODI-induced autophagy, we depleted ATG5 with a specific siRNA. Down-regulation of ATG5 inhibited 3′-ODI-induced autophagy in B16F1melanoma cells (Fig. 1E and F). The effect of ATG5 on 3′-ODI-induced autophagy was further addressed in ATG5 knockout mouse embryonic fibroblast (MEF) cells. Consistent with our previous data, ATG5-deficient MEF cells suppressed the conversion of LC3 protein by 3′-ODI treatment (Fig. 1G). These results suggest that 3′-ODI strongly induced autophagy and that ATG5 plays a key role in the activation of 3′-ODI-induced autophagy.

3.2. 3′-ODI inhibits α-MSH-stimulated melanin production in B16F1melanoma cells

The isoflavonoids have many properties such as anti-oxidant, anti-helminthic, anti-neoplastic, anti-inflammatory, and anti-melanogenic activities. To examine the cytotoxicity of 3′-ODI, we assessed cell viability. As shown in Fig. 2A, 3′-ODI was not cytotoxic to B16F1 melanoma cells at a concentration of 100 μM (Fig. 2A). To investigate the effect of 3′-ODI on melanin synthesis, B16F1 cells were stimulated with α-MSH and further treated with 3′-ODI. The results showed that α-MSH treatment strongly induced melanin synthesis. And co-treatment with 3′-ODI efficiently suppressed α-MSH-mediated over-production of melanin (Fig. 2B and C). We further evaluated the protein expression levels of key regulators of melanogenesis following 3′-ODI treatment. The expression levels of both tyrosinase and TRP1 were increased by α-MSH treatment. However, up-regulation of these proteins were remarkably reduced in 3′-ODI-treated cells, indicating that 3′-ODI inhibited α-MSH-stimulated melanin synthesis by down-regulation of melanogenesis regulators in B16F1 melanoma cells (Fig. 2D).

3.3. Inhibition of autophagy attenuates anti-melanogenic activity of 3′-ODI

Recent evidences have indicated that autophagy can participate in melanosome degradation and biogenesis [13,20]. In addition, we found that 3′-ODI induced autophagy but inhibited melanogenesis. Thus, we further addressed the effect of autophagy inhibition on melanin synthesis in 3′-ODI-treated cells. Interestingly, depletion of ATG5 by RNA interference notably reduced the anti-melanogenic effect of 3′-ODI in α-MSH-treated B16F1 melanoma cells (Fig. 3A). The effect of suppression of autophagy on 3′-ODI-mediated anti-melanogenesis was further addressed using key regulators of melanogenesis. Suppression of ATG5 recovered the protein levels of tyrosinase and TRP1 that were down-regulated by 3′-ODI in α-MSH-stimulated cells (Fig. 3B). These results suggest that autophagy regulates the anti-melanogenic effect of 3′-ODI in α-MSH-stimulated melanoma cells.

3.4. 3′-ODI-mediated autophagy regulates α-MSH-induced melanogenesis in melanocytes

The effect of 3′-ODI-mediated autophagy on melanogenesis was further confirmed in mouse Melan-a melanocytes. Similar to the B16F1 melanoma results, treatment with 3′-ODI efficiently induced autophagy activation in Melan-a cells (data not shown).

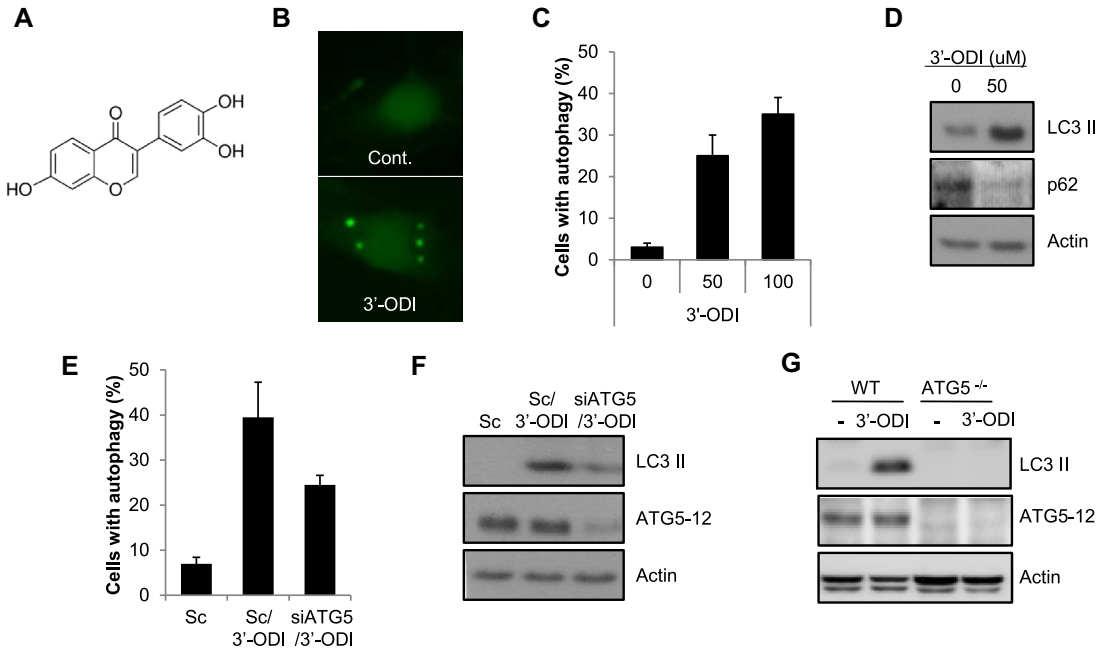

Fig. 1. 3′-ODI induces autophagy in B16F1 melanoma cells. (A) Chemical structure of 3′-ODI. (B) B16F1/GFP-LC3 cells were treated with 3′-ODI (100 μM) for 24 h. The cells were fixed and examined under a fluorescence microscopy. (C and D) B16F1/GFP-LC3 cells were exposed to increasing concentration of 3′-ODI (50, 100 μM). The cells with autophagic punctuate structure were counted under a fluorescence microscope. And the cells harvested to subject Western blot analysis with indicated antibodies (D). (E, F) B16F1/GFP-LC3 cells transfected with scrambled control siRNA (Sc) or ATG5 siRNA (siATG5) were treated with 3′-ODI (100 μM), and the autophagy activation was examined by counting of autophagic punctuate structure and conversion of LC3 protein. (G) Wild type MEFs (WT) or ATG5 knock-out MEFs (ATG5 −/−) cells were incubated with 3′-ODI (100 μM) for 24 h. And the autophagy activation was determined by detection of LC3 protein.

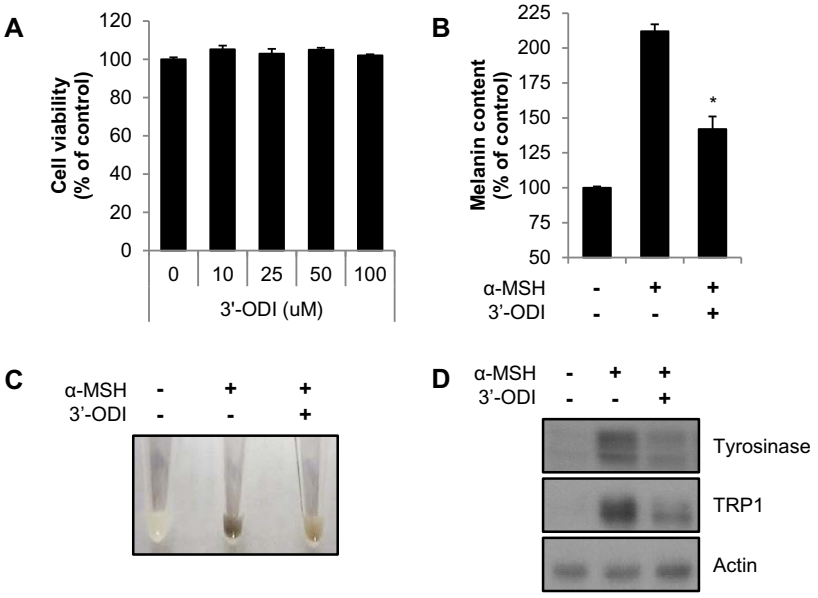

Fig. 2. 3′-ODI inhibits α-MSH-mediated melanogenesis in B16F1 melanoma cells. (A) After incubation of B16F1 cells with various concentrations of 3′-ODI for 24 h, the cell viability was determined with CCK-8 assay. (B–D) B16F1 cells pre-treated with α-MSH were further incubated with 3′-ODI (100 μM). After harvesting, the melanin content was measured as described in Section 2 (B), and the cell pellets were shown (C). The expression level of tyrosinase (TYR) and tyrosinase related protein-1 (TRP1) was analyzed by Western blotting. Data were obtained from least three independent experiments and values are presented as the means ± S.E.M. (*$p < 0.02$).

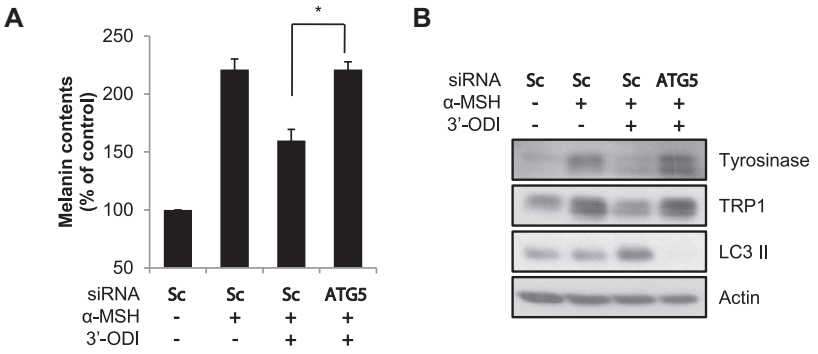

Fig. 3. Inhibition of autophagy attenuates 3′-ODI-mediated anti-melanogenic activity in B16F1 melanoma cells. (A, B) B16F1 cells were transfected with scrambled control siRNA and ATG5 siRNA. After 2 days, the cells were pre-treated with α-MSH (1 μM) for 24 h and exposed to 3′-ODI (100 μM) for additional 24 h. After 96 h from transfection, the cells were harvested to measure the melanin contents (A) and analyzed with Western blotting with indicated antibodies (B). Data represent ± standard error of the mean (S.E.M.) from more than three independent experiments).

Furthermore, the 3′-ODI-mediated down-regulation of melanin generation and 3′-ODI-mediated reduced tyrosinase and TRP1 protein levels were restored by the suppression of ATG5 in α-MSH-stimulated Melan-a melanocytes (Fig. 4A and B). Autophagy activation can be directly observed by electron microscopy (EM). Thus, the effect of 3′-ODI-mediated autophagy on melanogenesis was further investigated by EM analysis. Treatment with α-MSH induced accumulation of melanin in melanocytes (Fig. 4C). In addition, we identified autophagosomes that had engulfed melanin or melanosomes in α-MSH- and 3′-ODI-treated melanocytes (Fig. 4C), indicating that melanosomes can be degraded by autophagy. Taken together, these results suggest that autophagy mediates the anti-melanogenic effect of 3′-ODI in melanocytes.

4. Discussion

In this study, we selected phytochemicals that have an anti-melanogenic effect and generated a phytochemical library. From the library screening, we identified several previously known autophagy inducers, such as epigallocatechin gallate and resveratrol as well as 3′-ODI [21,22]. 3′-ODI treatment efficiently induced autophagy in melanoma cells and melanocytes (Figs. 1 and 4). In addition, 3′-ODI had strong anti-melanogenic activity in α-MSH-treated cells (Fig. 2). 3′-ODI suppressed hyperpigmentation through autophagy activity. Inhibition of autophagy by ATP5 depletion significantly suppressed 3′-ODI-mediated anti-melanogenesis as well as autophagy (Figs. 3 and 4).

3′-ODI is an isoflavone that is related to flavonoids, which are polyphenolic compounds wildely distributed in plants and have many functions in human health and are important factors for plant pigments. The most common source of isoflavone in food is soybeans, and the major isoflavones in soybeans are genistein and daidzein [19]. Previous studies have shown that several antioxidants negatively regulate melanogenesis. For instance, some flavonoids like quercetin prevent melanogenesis by inhibiting tyrosinase [23]. However, other flavonoids such as nobiletin and hesperetin stimulate melanogenesis by regulating cellular signaling [24,25]. Both genistein and daidzein have anti-oxidant properties, but the potential role of the anti-oxidant activity of 3′-ODI in autophagy-regulated melanogenesis is need to be further addressed [26,27].

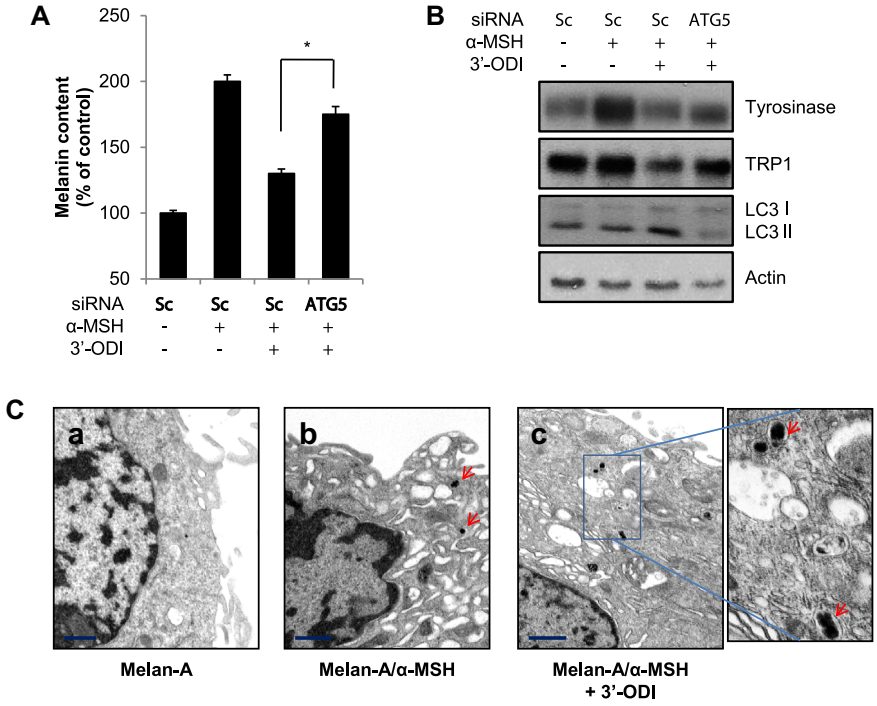

Fig. 4. Autophagy regulates 3′-ODI-mediated melanogenesis in Melan-a cells. (A, B) Melan-a cells were transfected with scrambled control siRNA and ATG5 siRNA. After 2 days, the cells pre-treated with α-MSH (1 μM) for 24 h were further exposed to 100 μM 3′-ODI for 24 h. Then, the melanin contents and expression of indicated protein were analyzed. (C) Melan-a cells and Melan-a cells treated with α-MSH (1 μM) were observed by electron microscopy as described in Section 2 (a, b). The red arrows indicate produced melanin (b). (c) Melan-a cells pre-treated with α-MSH were exposed to 3′-ODI (100 μM) for 24 h. Higher magnification electron microscopic picture of 3′-ODI treated cells shows autophagosomes (red arrows) that contain melanin. The scale bars present 1 μm. Data were obtained from least three independent experiments and values are presented as the means ± S.E.M. (*$p < 0.02$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Previously our group showed that *ortho*-dihydroxyisoflavone derivatives down-regulate α-MSH-stimulated melanogensis [15]. Here, we confirmed that 3′-ODI treatment notably suppressed not only α-MSH-induced accumulation of melanin but also α-MSH-induced up-regulation of tyrosinase and TRP1. MITF is the key regulatory transcription factor for melanogenesis-related proteins including tyrosinase and TRP1/2. Treatment of α-MSH up-regulates MITF via transactivation of the *MITF* gene by the cAMP signaling cascade. cAMP inhibits PI3K/AKT to activate *MITF* gene expression and increase transcriptional activity of MITF [28]. Seo et al. recently suggested that 3′-ODI suppresses the PI3K/AKT signaling pathway and reduces adipogenesis by ATP-competitive inhibition of PI3K in 3T3-L1 cells [29]. The AKT pathway positively regulates cell growth, proliferation, and survival. But AKT signaling is also known to suppress autophagy activation. AKT negatively regulates autophagy in response to mitogens by activation of mTOR, a critical autophagy regulator [30]. In addition, AKT can directly phosphorylate Beclin-1/ATG6 to suppress autophagy [31]. Thus, the role of AKT signaling in autophagy-mediated anti-melanogenic activity of 3′-ODI in α-MSH-stimulated cells remains to be further elucidated.

We showed that inhibition of autophagy by RNAi of ATG5 increased melanogenesis in melanoma cells as well as melanocytes (Figs. 3 and 4). Moreover, we found that chemical inhibitors for autophagy such as 3-methyladenin and Bafilmycin-A1 also up-regulated melanogenesis in B16F1 melanoma cells (data not shown). According to this notion, inhibition of autophagy by ULK1 depletion was shown to increase intracellular melanin contents in human melanoma MNT-1 cells [32]. van den Boorn et al. suggested that monobenzone, a skin depigmenting agent induced melanosome autophagy in human melanoma cells [20]. Autophagy is a cellular degradation process for unneeded organelles and cellular aggregates. Indeed, many cellular compartments including damaged mitochondria, ER, and peroxisomes are eliminated by the autophagy pathway. Melanin synthesized in melanosome is also an aggregate of smaller component molecules. Our electron microscopy data indicates that autophagosomes engulf over-produced melanin and melanosomes in α-MSH-stimulated cells (Fig. 4) and we assumed that the engulfed melanosomes by autophagosomes were degraded in a lysosome. Although a more detailed description of the molecular mechanisms involved in the regulation of melanogenesis by autophagy are needed, autophagy regulators may provide a novel experimental tool for the control of melanogenesis.

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