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Tranexamic acid inhibits melanogenesis by activating the autophagy system in cultured melanoma cells

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ABSTRACT

Background: As interest in skin beauty increases, the development of new skin whitening agents has attracted substantial attention; however, the action mechanism of the agents developed so far remains largely unknown. Tranexamic acid (TXA) is commonly being used to reduce melanin synthesis in patients with melasma and also used as a raw material for functional whitening cosmetics, although its action mechanism is poorly understood. Autophagy has been well known to be essential for tissue homeostasis, adaptation to starvation, and removal of dysfunctional organelles or pathogens. Recent studies have shown that autophagy regulators might have prominent roles in the initial formation stage of the melanosome, a lysosome-related organelle synthesizing melanin pigments. However, there is still no direct evidence showing a relationship between the activation of the autophagy system and the melanogenesis.

Objective: To investigate whether TXA can inhibit melanogenesis through the activation of autophagy in a melanoma cell line.

Methods: B16-F1 melanoma cells were treated with TXA and the levels of autophagy- and melanogenesis-related proteins were determined by Western blottings. The direct effect of TXA-mediated autophagy activation on melanin production was further evaluated by transfecting the cells with 60 pmols of small interfering RNAs (siRNAs)-targeting the mechanistic target of rapamycin (mTOR) and the autophagy-related protein 5 (Atg5).

Results: The results of Western blottings showed that TXA enhanced the production of autophagy-related proteins such as mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK) 1/2, Beclin-1, Atg12, and light chain 3 (LC3) I–II, whereas it decreased the synthesis of the mTOR complex. Confocal microscopy clearly showed that TXA treatment resulted in the formation of autophagosomes in B16-F1 cells, as revealed by immunostaining with an anti-LC3 antibody. The production of melanogenesis-associated proteins, including microphthalmia-associated transcription factor (MITF), tyrosinase, and tyrosinase-related protein 1 and 2 (TRP1/2), were clearly downregulated by the treatments with TXA. These results suggest that TXA can mediate a decrease in melanin synthesis by alleviating the production of tyrosinase and TRP1/2, along with lowered MITF protein levels. Furthermore, after treatment with TXA, siRNAs- targeting to mTOR and Atg5 increased melanin synthesis by 20% and 40%, respectively, compared to that in non-transfected cells, in a dose-dependent manner. These results further confirmed that TXA can inhibit melanogenesis by activating the autophagy system.

Conclusion: Collectively, the results demonstrate that TXA can reduce melanin synthesis in melanoma B16-F1 cells by activating the ERK signaling pathway and the autophagy system.

Abbreviations: α-MSH, α-melanocyte-stimulating hormone; ERK, extracellular signal-regulated kinase; DHICA, 5,6-dihydroxyin-dole-2-carboxylic acid; LC3, light chain 3; L-DOPA, L-3,4-dihydroxyphenylalanine; mTOR, mechanistic target of rapamycin; MITF, microphthalmia-associated transcription factor; TGF-β, transforming growth factor-beta; TRP1/2, tyrosinase-related protein 1/2; TXA, tranexamic acid; uPA, urokinase-type plasminogen activator; UV, ultraviolet.

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1. Introduction

The colors of the skin, hair, and eye are derived from the biological pigmentation factor melanin, which is produced via melanogenesis within melanosomes [1]. The amount of melanin produced is influenced by various genetic and environmental factors, including exposure to ultraviolet (UV) light. One of the roles of melanin is to protect the skin, tissues, and genes from UV-induced skin injury [2]. In response to UV light, preexisting melanin pigments in melanocytes are immediately redistributed, which is followed by an enhanced melanogenesis in epidermal melanocytes and increased the transfer of melanin to epidermal keratinocytes [3]. However, the formation of excessive melanin in the skin results in hyperpigmentation, which can induce skin disorders, such as melasma, freckles, and geriatric pigment spots [4]. Therefore, studies regarding the regulation and mechanism of melanogenesis are important to identify targets for the prevention and treatment of hyperpigmentation disorders.

Melanin is initially produced in two different forms, eumelanin and pheomelanin, in which tyrosinase and tyrosinase-related protein 1/2 (TRP1/2) are involved as main factors. In the melanogenesis, tyrosinase is a rate-limiting enzyme that catalyzes the conversion of tyrosine to L-3,4-dihydroxyphenylalanin (L-DOPA) and subsequently oxidizes this molecule to form dopaquinone. Eumelanin is synthesized via TRP-2, which functions as a DOPA chrome tautomerase and catalyzes the rearrangement of DOPA chrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA); subsequently, TRP-1 oxidizes DHICA to its carboxylated form, indole-quinone [5]. Pheomelanin is synthesized through a reaction involving dopaquinone and cysteine. In addition, tyrosinase and TRP1/2 are transcriptionally regulated by microphthalmia-associated transcription factor (MITF) in melanocytes [6]. The phosphorylation of MITF by extracellular signal-regulated kinase (ERK) 2 results in the degradation of the former via the proteasome-mediated proteolytic pathway [1].

Cellular autophagy system is well known to play an important role in removing waste proteins (misfolded or aggregated proteins) and also in eliminating dysfunctional organelles, including the mitochondria, endoplasmic reticulum, and peroxisomes [5,7]. Recent studies show that the autophagy system might also be involved in the biogenesis of melanin and the degradation of the melanosome, a lysosome-related organelle synthesizing melanin pigments, suggesting that its activation can be closely related to skin color by reducing the production of melanin [8]. In fact, some studies have shown that autophagy regulators may have prominent roles in the initial stages of melanosome formation [9,10]. Beclin-1 [11], autophagic modulator WIPI1 (a mammalian homolog of ATG18) [12], and microtubule-associated protein light chain 3 (LC3) are potent regulators for the melanogenesis in melanocytic lesions [13]. In addition, a factor called mechanistic target of rapamycin (mTOR) acts as a critical inducer for autophagy system, as its positive regulation suppresses this process through Akt and mitogen-activated protein kinase (MAPK) signaling pathway; however, its negative regulation executed by AMPK and the p53 signaling pathway promotes autophagy [14,15]. The ERK1/2 pathway also plays a key role in regulating autophagy [1,16].

Tranexamic acid (TXA; *trans*-4-aminomethylcyclohexanecarboxylic acid) has been developed initially as a plasmin inhibitor that can be used for treating a heavy bleeding resulted from trauma, surgery, and menstruation [17]. The compound is a synthetic derivative of the amino acid lysine and exerts its effects by reversibly blocking lysine-binding sites on plasminogen molecules [18]. TXA has also emerged as a potential drug for treating melasma, since it can inhibit the melanin synthesis by inhibiting the plasminogen/plasmin pathway, thereby blocking the interaction between melanocytes and keratinocytes [19]. As plasminogen also exists in cultured human keratinocytes, which are known to produce plasminogen activators, it can be expected that TXA may have an effect on the function of keratinocytes [20]. However, the action mechanism of TXA related to its direct involvement in melanogenesis in view of signaling pathway(s) has not yet been studied in detail.

Based on the results from previous studies, we postulated that there would be a mechanistic link between autophagy activation and melanogenesis, which might help to understand the action mechanism of TXA related to its suppressive ability against melanin synthesis. We describe here a relationship between the melanogenesis and the activation of autophagy as well as how TXA involves in inhibiting the melanin synthesis by activating cellular autophagy system in cultured B16-F1 melanoma cell line. The results obtained by the present study demonstrate that TXA has a great potential of becoming a functional agent for the treatment of pigment disorders as well as a cosmetic product for skin whitening.

2. Materials and methods

2.1. Materials

The compound TXA was a kind gift from a Korean company Hugel (Chuncheon, Korea). Mushroom tyrosinase, arbutin, poly-L-lysine (0.01% solution), 1% penicillin-streptomycin, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified eagle's medium (DMEM) was from Lonza (Walkersville, MD, USA). Antibodies raised against MAP-LC3β, GAPDH, tyrosinase, and TRP1 were purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and Beclin-1, Atg12, phospho-Erk1/2, p44/42 MAP kinase, phospho-p38 MAP kinase, p38 MAPK, MITF, phospho-mTOR, and mTOR were from Cell Signaling Technology (Danvers, MA, USA).

2.2. Cell culture

Murine melanoma B16-F1 cells were purchased from the Korean Cell Line Bank (KCLB; Seoul, Korea) and routinely used by this study. The cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS; ATLAS Biologicals, Fort Collins, CO, USA) and 1% penicillin-streptomycin (Sigma-Aldrich; St. Louis, MO, USA) at 37 °C in 5% CO_2 incubator. The culture medium was changed every 2 days [2] and the cells were harvested by trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Welegen; Daegu, Korea) when they reached to approximately 70% confluency.

2.3. Cell viability assay

Cell viability was evaluated using the CellTiter 96® Non-Radioactive cell proliferation assay (Promega; Madison, WI, USA). B16-F1 cells cultured at a density of 0.5×10^5 cells/well in 96-well plates for 24 h were treated with various concentrations of TXA (0.1–10 mg/ml), arbutin (0.1–10 mg/ml), and TGF-β (0.00002–0.2 ng/ml) for 24 h. At the end of the incubation, 15 μl of MTT dye solution was added to each well and further incubated at 37 °C in 5% CO_2 for 4 h. After adding 100 μl of the Solubilization Solution/Stop Mix per well, the reaction was continued for 1 h and the absorbance at 570 nm was measured using a 96-well plate reader.

2.4. Western blot analysis

B16-F1 cells were seeded at a density of 0.5×10^5 cells/well in 6-well culture plates. After incubating for 24 h, the cells were treated with TXA (1 mg/ml) or TGF-β (10 ng/ml) to induce autophagy for 15 min. In addition, to induce melanogenesis, the cells were treated

with α-MSH (10 nM), together with TXA (1 mg/ml) or arbutin (0.2 mg/ml) for 24 h at 37 °C. Cell lysates were then prepared using a ProNA CETi lysis buffer (TransLab; Daejeon, Korea) and the protein concentrations were measured by Bradford assay as describe elsewhere. For SDS-PAGE, normally 50 μg of proteins were boiled at 100 °C for 3 min and then loaded onto 8%, 12%, or 15% polyacrylamide gel [1]. The electrophoresed proteins were then transferred onto PVDF membrane (Bio-Rad; Hercules, CA, USA) and blocked with 5% skim milk in TBS-T (250 mM Tris-HCl, 1.5 mM NaCl, 0.1% Tween 20, pH 8.0) at room temperature (RT) for 2 h. The membrane was then incubated with primary antibodies (1:500 diluted in the blocking buffer) overnight at 4 °C. After washing six times with TBS-T buffer, the membrane was then incubated with HRP-conjugated secondary antibodies (1:4000 in the blocking buffer) at RT for 2 h and washed five times with TBS-T buffer. The signals were detected using EZ-Western Lumi Plus system (DaeilLab Service; Seoul, Korea).

2.5. Immunostaining for confocal microscopic analysis

B16-F1 cells were seeded onto glass coverslips coated with 0.1% poly-L-lysine (Sigma-Aldrich; St. Louis, MO, USA) at a density of 0.1×10^5 cells per well in 12-well culture plates. After culturing for 24 h, the cells were treated with TXA (1 mg/ml) for 15 min or TGF-β (10 ng/ml) for 30 min in the absence or presence of α-MSH (10 nM). The cells were then fixed with 3.7% formaldehyde for 20 min at RT, permeabilized with 0.1% TritonX-100 for 10 min, and blocked with 1% BSA for 20 min. After washing several times with PBS, the cells were incubated with anti-LC3 bodies (diluted 1:50 in PBS) overnight at 4 °C and treated with Alexa Fluor 488-conjugated goat anti-mouse IgGs diluted 1:200 in PBS as secondary antibodies (Invitrogen; Carlsbad, CA, USA). The cells were finally stained with 5 μl of a solution called Prolong Gold Antifade with DAPI purchased from Life Technologies (Grand Island, NY, USA) and the resulting images were observed using a LSM-510 microscope (Carl Zeiss; Thornwood, NJ, USA).

2.6. Transfection of siRNAs

B16-F1 cells were transfected with 60 pmols each of small interfering RNAs (siRNAs) specific to mTOR and Atg5 mRNAs that were obtained from Santa Cruz Biotechnology (Dallas, TX, USA) in a siRNA transfection reagent and suspended in a transfection medium (serum-free medium) at 37 °C for 24 h in 5% CO_2 incubator. To examine the rate of melanin synthesis, the non-transfected control and transfected cells were used as described previously [21].

2.7. Measurement of melanin content

B16-F1 cells were cultured at a density of 0.5×10^5 cells/well in 6-well plates using the DMEM without phenol red (Welgene; Daegu, Korea). After culturing for 24 h, the cells were treated with 10 nM of α-MSH (Sigma-Aldrich; St. Louis, MO, USA) alone or 10 nM of α-MSH plus various concentrations of TXA (0.1, 0.5, 1, 2, and 4 mg/ml) for 52 h at 37 °C. The resulting culture supernatant was collected by centrifuging at approximately $16{,}000 \times g$ for 4 min and the absorbance at 490 nm was measured, in which a standard graph was made using synthetic melanin (0 to 50 μg/ml) (Sigma-Aldrich; St. Louis, MO, USA).

2.8. Determination of the whitening efficacy of TXA on 3D human skin model

A 3D human skin melanoderm was purchased from the Mat-Tek Corporation (Ashland, MA, USA) and used for evaluating TXA's whitening efficacy, which was done by Molecular Skin Biotechnology Laboratory, Kyunghee University (Seoul, Korea).

3. Results and discussion

3.1. Activation of the autophagy system by TXA in B16-F1 cells

To examine the effect of TXA on the activation of autophagy, the expressions of molecules related to the ERK1/2 pathway were

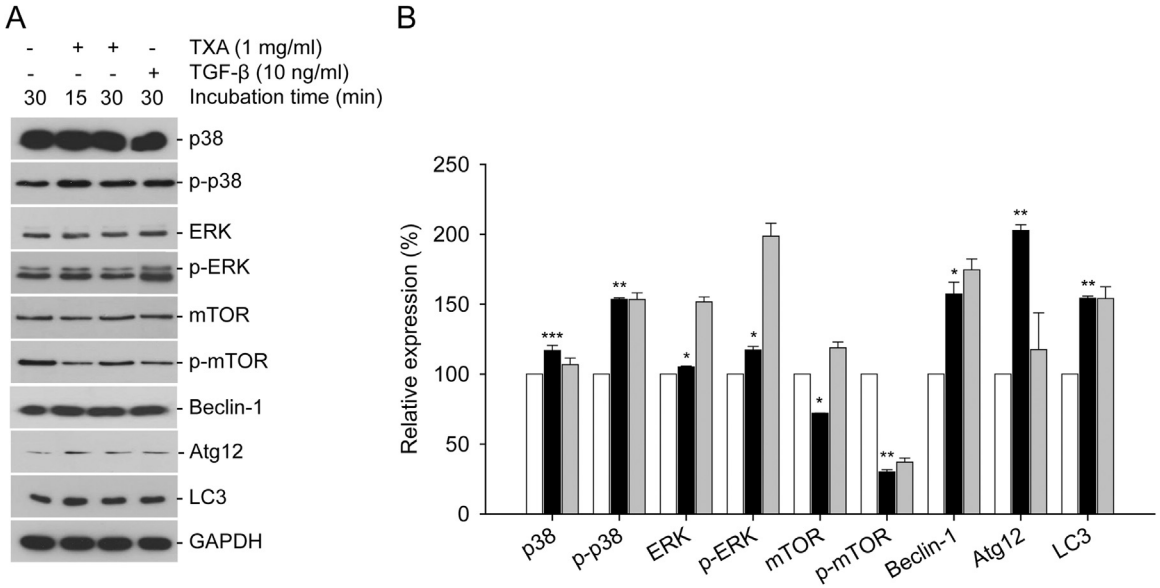

Fig. 1. Induction of autophagy by TXA through the activation of MAPK/ERK1/2 signaling pathway. (A) B16-F1 cells were treated with TXA (1 mg/ml) for 15 or 30 min, or with TGF-β (10 ng/ml) for 30 min as a positive control. Proteins were separated by SDS-PAGE and Western blottings were performed using antibodies raised against p38, p-p38, ERK1/2, p-ERK1/2, mTOR, p-mTOR, Beclin-1, Atg12, LC3, and GAPDH. The symbols "−" and "+" indicate the omission and the addition, respectively. (B) The relative expression levels of p38, p-p38, ERK1/2, p-ERK1/2, mTOR, p-mTOR, Beclin-1, Atg12, and LC3 at 15 min with TXA treatment (black colors) and at 30 min with TGF-β treatment without TXA (grey colors) are shown by histograms. White colored histograms show the non-treated control groups. Each value was calculated as the ratio of signal intensity normalized to that of GAPDH. The values were expressed as mean ± SD from the triple Western blots. *, $P < 0.001$; **, $P < 0.0001$; ***, $P < 0.01$ verse compared to that of the untreated control group.

examined in B16-F1 cells after treatment with TXA. When the cells were treated with TXA (1 mg/ml) for 15 min or TGF-β (10 ng/ml) for 30 min as a positive control, the expression levels of phospho-p38, phospho-ERK1/2, Beclin-1, Atg12, and LC3 were increased by approximately 46%, 17%, 48%, 126%, and 49%, respectively, compared to their levels in the untreated control groups, as revealed by Western blottings (Fig. 1). However, TXA down-regulated the expression of p-mTOR that is a typical negative regulator of autophagy by approximately 65%, compared to that of non-treated control group. These results suggest that TXA can induce the upregulation of autophagy-related proteins, including Beclin-1, Atg12, and LC3 in B16-F1 cells by activating the ERK1/2 signaling pathway.

3.2. Formation of the autophagosomes by TXA

The ability of TXA to enhance the formation of autophagosome was examined using confocal microscopy after immunostaining with an anti-LC3 antibody, as the molecule LC3 is a typical marker for the formation of autophagosome. As shown in Fig. 2A, autophagosomes could be observed clearly in the cells treated with TXA (1 mg/ml) for 15 min as in the cells treated with TGF-β (10 ng/ml) for 30 min as a positive control. As expected, autophagosomes appeared more clearly in the cells treated with the same concentrations of TXA and TGF-β for the same duration time periods used in Fig. 2A, in the presence of α-MSH (10 nM) that is well-known as one of melanotropins stimulating melanogenesis [2]. These results suggest that TXA can lead the formation of the autophagosomes.

3.3. Inhibitory effect of TXA on melanogenesis

It has been well established that the phosphorylation of ERK1/2 results in the degradation of MITF, which in turn downregulates the expression of melanogenesis-associated proteins such as tyrosinase and TRP1/2, leading to the suppression of melanogenesis [22]. Therefore, it was necessary to determine the action mechanism of TXA on melanogenesis in terms of its ability to activate the ERK signaling pathway, together with its effects on the expressions of MITF, tyrosinase, and TRP-1 by Western blottings. As shown in Fig. 3A and B, the expression levels of ERK1/2 and p-ERK1/2 were increased to 18% and 5%, respectively, when B16-F1 cells were treated with 10 nM of α-MSH. In contrast, the levels of MITF, tyrosinase, and TRP 1 were significantly decreased to 17%, 14%, and 5%, respectively, compared to that of non-treated control when the cells were treated with 1 mg/ml of TXA for 24 h in the presence of α-MSH. These results suggest that TXA has a synergic action mechanism to exert its anti-melanogenic effect: (1) it can activate the ERK signaling pathway, which leads to the degradation of MITF, which, in turn, down-regulates the expression of melanogenesis-associated proteins, including tyrosinase and TRP1/2; (2) it can also directly suppress the production of the same proteins and enzymes, including MITF, tyrosinase, and TRP 1/2.

3.4. Inhibitory effect of TXA on melanin synthesis

As mentioned, TXA can act as an inhibitor against urokinase-type plasminogen activator (uPA) and also inhibit the melanin synthesis in melanocytes through by interfering with the interaction between melanocytes and keratinocytes [23,24]. In this study, a possible inhibitory effect of TXA on melanin production was also examined in B16-F1 cells. To investigate the effect of TXA on melanin production, the cells were treated with various concentrations of TXA (0.1, 0.5, 1, 2, or 4 mg/ml) for 52 h in the presence 10 nM of α-MSH. As shown in the control group of Fig. 4A, the rates of melanin production were clearly decreased by the treatments with TXA in dose-dependent manners; in particular, the treatment of 4 mg/ml of TXA resulted in the decrease of the melanin synthesis to approximately 46%, compared to that observed in cells treated with α-MSH only. The skin whitening effect by TXA through by its suppressive ability to the melanin

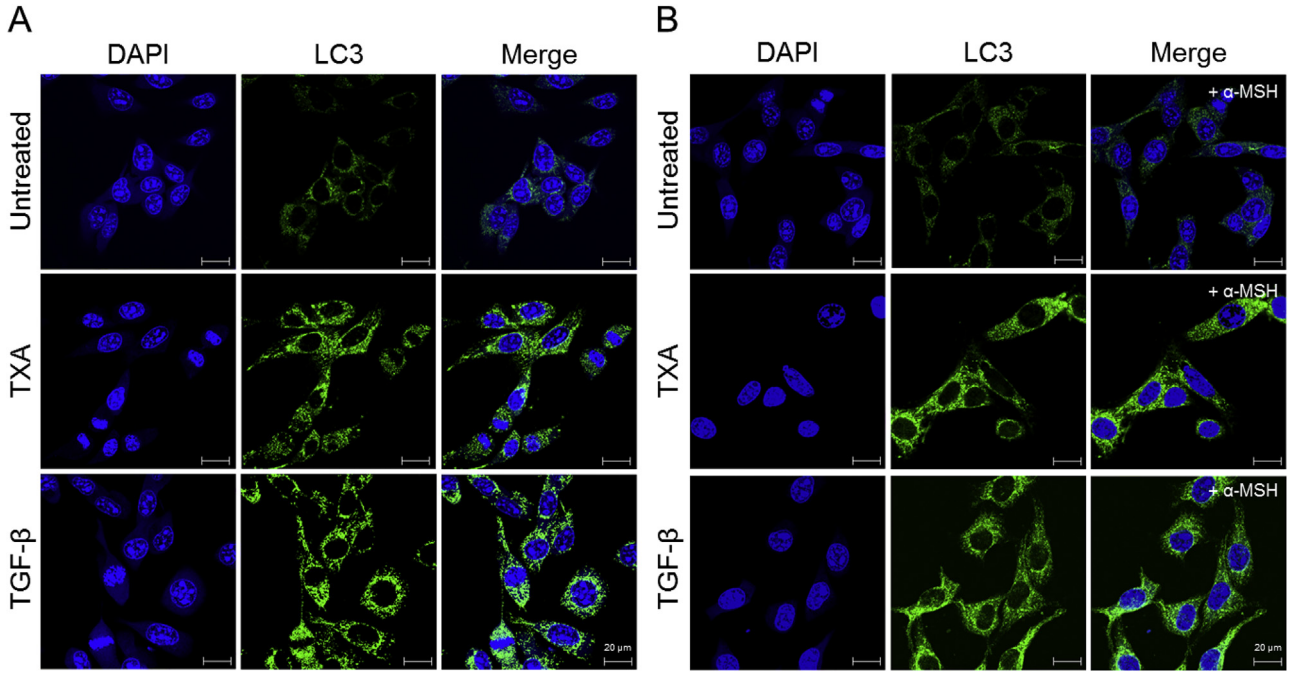

Fig. 2. Confocal microscopic analysis of the formation of autophagosomes by TXA. (A) Cells treated with TXA (1 mg/ml) for 15 min or TGF-β (10 ng/ml) for 30 min in the absence of α-MSH were stained with anti-LC3 antibody labeled with fluorescein, and the resulting green fluorescence was observed using a confocal microscope. (B) The cells treated with TXA (1 mg/ml) for 15 min or TGF-β (10 ng/ml) for 30 min in the presence of α-MSH (10 nM) were stained with the same anti-LC3 antibody and the fluorescence was observed using the same microscope.

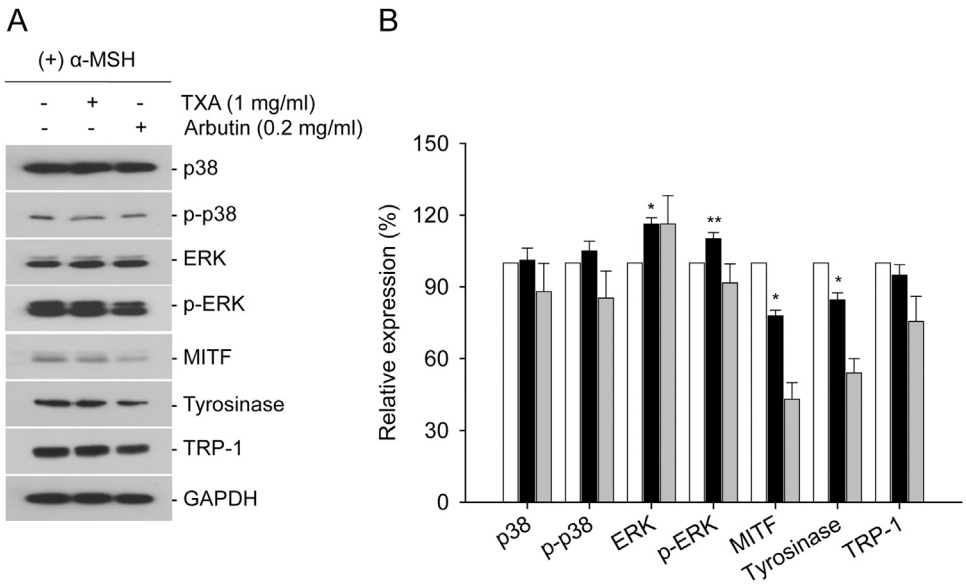

Fig. 3. Effect of TXA on ERK1/2 signaling and melanogenesis-associated protein expression. (A) Cells were treated with TXA (1 mg/ml) or arbutin (0.2 mg/ml) as a positive control for 24 h. Proteins from each sample were separated by SDS-PAGE and Western blottings were performed with antibodies raised against p38, p-p38, ERK1/2, p-ERK1/2, MITF, tyrosinase, TRP-1, and GAPDH. The symbols "−" and "+" indicate the omission and the addition, respectively. (B) The relative expression levels of p38, p-p38, ERK1/2, p-ERK1/2, MITF, tyrosinase, and TRP-1 are shown, in which black- and grey-colored histograms indicate the data obtained from the TXA and arbutin treatment groups, respectively. White colored histograms show the non-treated control groups. Each value was calculated as the ratio of signal intensity normalized to that of GAPDH. The values were expressed as mean ± SD from the triple Western blots. *, $P < 0.001$; **, $P < 0.005$ verse the untreated control group.

synthesis could also be observed in a melanoderm 3D skin tissue model for 12 days, in which the degree of TXA's whitening efficacy was estimated to approximately twice stronger than a compound called arbutin that is known to have good whitening agent (see the Supplementary Fig. 1). All these results strongly suggest that TXA has an ability to inhibit the melanin synthesis in melanoma cell line and skin tissue as well.

3.5. Effect of siRNAs specific to mTOR and Atg5 on melanin synthesis

The results described above demonstrate that TXA can activate the autophagy system by upregulating autophagy-related proteins, and can inhibit melanogenesis by stimulating the ERK signaling pathway and by directly downregulating melanogenesis-associated proteins. However, it was still necessary to confirm that TXA can activate autophagy, eventually leading to the reduction of melanin synthesis, by using a reverse-genetics approach with siRNAs that are targeting to mTOR and Atg5 mRNAs. As shown in Fig. 4A, there were clear decreases in melanin synthesis dose-dependently, when non-transfected cells were treated with various concentrations of TXA (0.1, 0.5, 1, 2, or 4 mg/ml) for 52 h. As expected, the same results could be observed from Mock groups as well. However, the inhibitory effect of TXA on the melanin synthesis were relieved significantly in the cells transfected with the siRNAs specific to mTOR and Atg5 for 52 h (Fig. 4A). In addition, the melanin content analysis clearly showed that the rates of melanin synthesis by mTOR and Atg5 siRNAs were increased to approximately 20% and 40%, respectively, when the

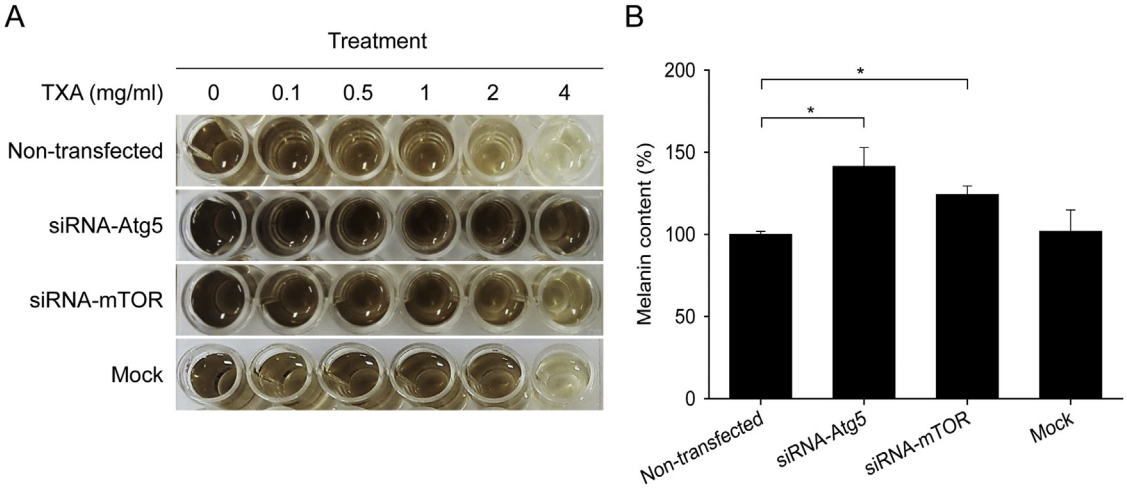

Fig. 4. Effect of mTOR- and Atg5-knockdowns on melanin production as indicated. Cells were non-transfected or transfected with siRNAs specific for mTOR, Atg5 or a non-specific control (Mock) siRNA. (A) Cells were co-treated with α-MSH (10 nM) and TXA (0.1–4 mg/ml) for 52 h and the plate was photographed to visualize. (B) The non-transfected and transfected cells as indicated were treated with TXA (4 mg/ml) for 52 h in the presence of α-MSH (10 nM) and the melanin content analyses were performed as described in Materials and methods. Data are presented as mean ± SD, in which the data from non-transfected control groups treated with 4 mg/ml of TXA are regarded as a value of 100%. *, $P < 0.0001$.

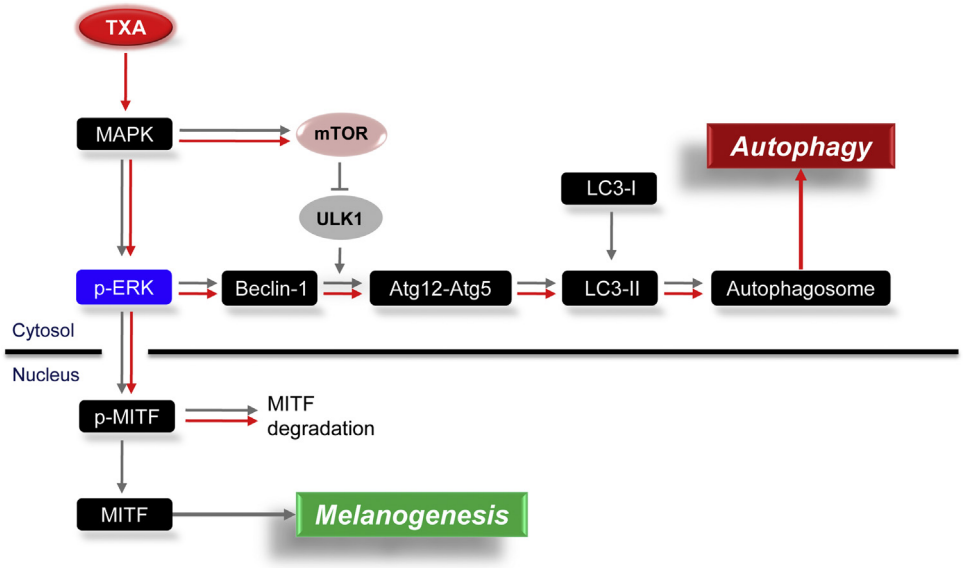

Fig. 5. Summary of TXA's inhibitory action mechanism on melanogenesis in part. TXA activates the ERK signaling pathway, leading to the activation of the autophagy system, which results in the degradation of MITF, which now in turn downregulates the expression of melanogenesis-associated proteins, including tyrosinase and TRP1/2. The signaling pathways that are already known and triggered by TXA are shown in gray- and red-colored arrows, respectively.

transfected cells were treated with 4 mg/ml of TXA for 52 h, compared to that in non-transfected cells (Fig. 4B).

4. Conclusions

Collectively, as shown in Fig. 5 the results obtained by this study demonstrate that TXA has the following effects: (1) it can activate the autophagy system by upregulating autophagy-related proteins, including Beclin-1, Atg12, and LC3, through by inhibiting the expression of mTOR; (2) it can activate the ERK signaling pathway, resulting in the degradation of MITF, which in turn downregulates the expression of melanogenesis-associated proteins such as tyrosinase and TRP1/2; (3) it can exert a direct suppressive role in the productions of melanogenesis-related proteins and enzymes. These results clearly show that TXA can regulate melanin synthesis in B16-F1 cells through the activation of the autophagy system. Therefore, the results show that TXA could be used as a potential agent for melasma treatment and as a cosmetic compound for skin whitening.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jdermsci.2017.05.019.

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