

Involvement of Transglutaminase-2 in α -MSH-Induced Melanogenesis in SK-MEL-2 Human Melanoma Cells

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Abstract

Skin hyperpigmentation is one of the most common skin disorders caused by abnormal melanogenesis. The mechanism and key factors at play are not fully understood. Previous reports have indicated that cystamine (CTM) inhibits melanin synthesis, though its molecular mechanism in melanogenesis remains unclear. In the present study, we investigated the effect of CTM on melanin production using ELISA reader and the expression of proteins involved in melanogenesis by Western blotting, and examined the involvement of transglutaminase-2 (Tgase-2) in SK-MEL-2 human melanoma cells by gene silencing. In the results, CTM dose-dependently suppressed melanin production and dendrite extension in α -MSH-induced melanogenesis of SK-MEL-2 human melanoma cells. CTM also suppressed α -MSH-induced chemotactic migration as well as the expressions of melanogenesis factors TRP-1, TRP-2 and MITF in α -MSH-treated SK-MEL-2 cells. Meanwhile, gene silencing of Tgase-2 suppressed dendrite extension and the expressions of TRP-1 and TRP-2 in α -MSH-treated SK-MEL-2 cells. Overall, these findings suggested that CTM suppresses α -MSH-induced melanogenesis via Tgase-2 inhibition and that therefore, Tgase-2 might be a new target in hyperpigmentation disorder therapy.

Key Words: Cystamine, Melanogenesis, Transglutaminase-2, TRP-1, TRP-2, SK-MEL-2 melanoma cells

INTRODUCTION

Skin pigmentation consists of the synthesis and transfer of melanin by epidermal melanocytes to the governing keratinocytes (Yamaguchi *et al.*, 2007). This skin pigmentation may result from several steps including melanocyte proliferation, differentiation, melanogenesis, migration or increases in dendricity (Park *et al.*, 2009). In melanocytes, melanins are synthesized within melanosomes which contain three major pigment enzymes: tyrosinase, tyrosinase-related protein (TRP-1), and TRP-2 (Lee *et al.*, 2012b). The expression of these proteins is regulated transcriptionally by the microphthalmia associated transcription factor (MITF). MITF is a unique, important transcription factor involved in all aspects of melanocyte survival and function (Lee *et al.*, 2011).

Skin hyperpigmentation is the most common skin disorder caused by sun damage, inflammation, or other skin injuries, including those related to acne vulgaris (Lim *et al.*, 2009; Chang and Chen, 2012). Disorders of hypopigmentation include al-

binism, piebaldism, tuberous sclerosis, hypomelanosis of Ito, vitiligo, pityriasis alba, tinea versicolor and post-inflammatory hypopigmentation (Plensdorf and Martinez, 2009). Although various compounds and physical therapies have been developed over the past decades for the treatment of skin pigmentation disorder, none is completely satisfactory.

α -melanocyte-stimulating hormone (α -MSH) is a naturally-occurring endogenous peptide of the melanocortin family that stimulates melanin synthesis and its distribution in melanocytes (in mammals) and melanophores (in lower vertebrates) (Brzoska *et al.*, 2008). α -MSH exerts its effect on pigmentation by binding the melanocortin-1 receptor (MC1R) at the cell membrane of melanocytes (Yang *et al.*, 1997).

Cystamine (CTM) is an organic disulfide known to inhibit transglutaminase 2 (Tgase-2) activity via a disulfide exchange process (Borrell-Pages *et al.*, 2006). CTM is involved in melanin synthesis via the reduction of the tyrosinase (TYR) activity of pigmented melanoma cells (Qiu *et al.*, 2000). However, the exact molecular mechanism of CTM in melanogenesis is cur-

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rently unknown.

Tgase-2 is a multifunctional protein having both intracellular and extracellular functions (Lee *et al.*, 2012a). In addition to catalyzing Ca^{2+} -dependent transamidation reactions, Tgase-2 can bind and hydrolyze GTP/GDP with an affinity and catalytic rate similar to those of the α subunit of large heterotrimeric G proteins and small Ras-type G proteins (Lorand and Graham, 2003; Mhaouty-Kodja, 2004; Lee *et al.*, 2012a). Interestingly, it has been shown that metastatic melanoma cell lines express Tgase-2 levels up to 24-fold higher than do primary melanoma cell lines in the radial growth phase (Fok *et al.*, 2006). The involvement of Tgase-2 in human melanogenesis, however, has not been reported. Therefore, in the present study, using gene silencing and CTM, we investigated the involvement of Tgase-2 in melanogenesis. In this report, we found that transglutaminase-2 is involved in α -MSH-induced melanogenesis of SK-MEL-2 melanoma cells.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS) and culture media were obtained from WelGENE Inc (Daegu, South Korea). CTM and α -melanocyte-stimulating hormone (α -MSH) were purchased from Sigma Aldrich (St. Louis, MO, USA). All other chemicals used were of standard analytical grade. TRP-1, TRP-2, and β -actin antibodies were acquired from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Transglutaminase 2 (Tgase-2) monoclonal antibody (clone CUB 7402) was purchased from NeoMarkers (Fremont, CA, USA). siRNAs and control siRNAs were obtained from Invitrogen (Carlsbad, CA, USA).

Cell culture

SK-MEL-2 cells were purchased from Korean Cell Line Bank (KCLB). The cells were grown and maintained at 37°C in a 95% air, 5% CO_2 atmosphere in RPMI-1640 supplemented to a final concentration of 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. Cells were passaged every 3 days with a maximal passage number of 33. Confluent monolayers of melanocytes were harvested with a mixture of 0.05% trypsin and 0.53 mM EDTA (Bibco BRL, Grand Island, NY, USA).

Melanin measurement

The melanin content of the cultured melanoma was determined as described previously (Kim *et al.*, 2010). Preparatory to cellular melanin measurement, cells were seeded in a 6-well plate at an appropriate density, and, after 24 hrs of cultivation, were treated with α -MSH and various concentrations of CTM for 48 hrs. The cells were then harvested, washed twice with PBS, resuspended in 1 N NaOH containing 10% DMSO, and heated at 80°C for 1 hr. Subsequently, the absorbance of extracted melanin was read at 405 nm using an ELISA microplate reader.

Western blot analysis

After incubation, cells were collected and washed twice with cold PBS. The cells were then lysed in a lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% triton X-100, 2 mM EDTA, 1% DOC (Deoxycholic acid), 0.1% SDS, 1 mM NaVO_3 , 10 mM NaF, 1 mM DTT] and centrifuged to yield whole-cell ly-

sates, the protein concentration of which was measured using the Bradford method. Aliquots of the lysates (20-30 μg of protein) were separated on 4-12% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (Invitrogen) with a glycine transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH 8.8), 10% MeOH (v/v)]. After blocking the non-specific site with 3% non-fat dry milk, the membrane was then incubated with specific primary antibody in 3% BSA at 4°C overnight, and then further incubated with peroxidase-conjugated secondary antibody (1:5000, Santa Cruz, CA, USA) at room temperature for 60 min. Immunoactive proteins were detected using the PowerOpti-ECL Western blotting detection reagent (Animal Genetics Inc., Gyeonggi, Korea).

Chemotactic migration assay

Migration assays were performed using multiwell chambers (Neuroprobe Inc. Gaithersburg, MD, USA) coated with 10 $\mu\text{g}/\text{ml}$ fibronectin as a chemoattractant. Briefly, the cells were suspended at 1×10^6 cells/ml in DMEM (2% FBS), a 25 μl aliquot of which was poured into the upper well of a chamber. Next, the aliquot was separated from the sample-containing lower well by means of an 8 μm polyhydrocarbon filter. After incubation for 5 hrs at 37°C, non-migrated cells on the upper surface of the membrane were scraped off, and the migrated cells on lower surface were stained by Diff-quick and subsequently counted under four randomly chosen high-power fields (400 \times).

Cell proliferation assay

The cell proliferation was measured using the EZ-Cytox Cell viability assay kit (Daeilab Service, Seoul, Korea). Briefly, 100 μl of cell suspension (3000 cells per well) was added to each well of a 96-well plate. After the required incubation with stimulants for 48 hrs, 10 μl of EZ-Cytox solution was added to each well of the plate and incubated at 37°C for 2-4 hrs. The absorbance was measured by spectrophotometry (Multiscan, Thermo, USA) at 450 nm. The cell proliferation (%) was calculated using the formula $[A_s/A_c] \times 100\%$, where A_s is the absorbance of the well-contained cells, culture medium, EZ-Cytox solution and stimulants, and A_c is the absorbance of the well-contained cell, culture medium and EZ-Cytox solution.

Tgase-2 gene silencing by small-interfering RNA

A small-interfering RNA (siRNA) duplex-targeting human Tgase-2, 5'-AAG AGC GAG AUG AUC UGG AAC-3' (Invitrogen), was introduced into the cells using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions. The cells were then cultured with or without α -MSH (100 nM). Universal negative siRNA (Invitrogen) was employed as a negative control.

Statistical analysis

An analysis of variance (ANOVA) in conjunction with Tukey's post-hoc test was used to determine the statistical significance of the differences between the values for a variety of experimental and control groups. The data are expressed as mean \pm s. d. of at least three independent experiments performed in triplicate. $p < 0.05$ was considered statistically significant.

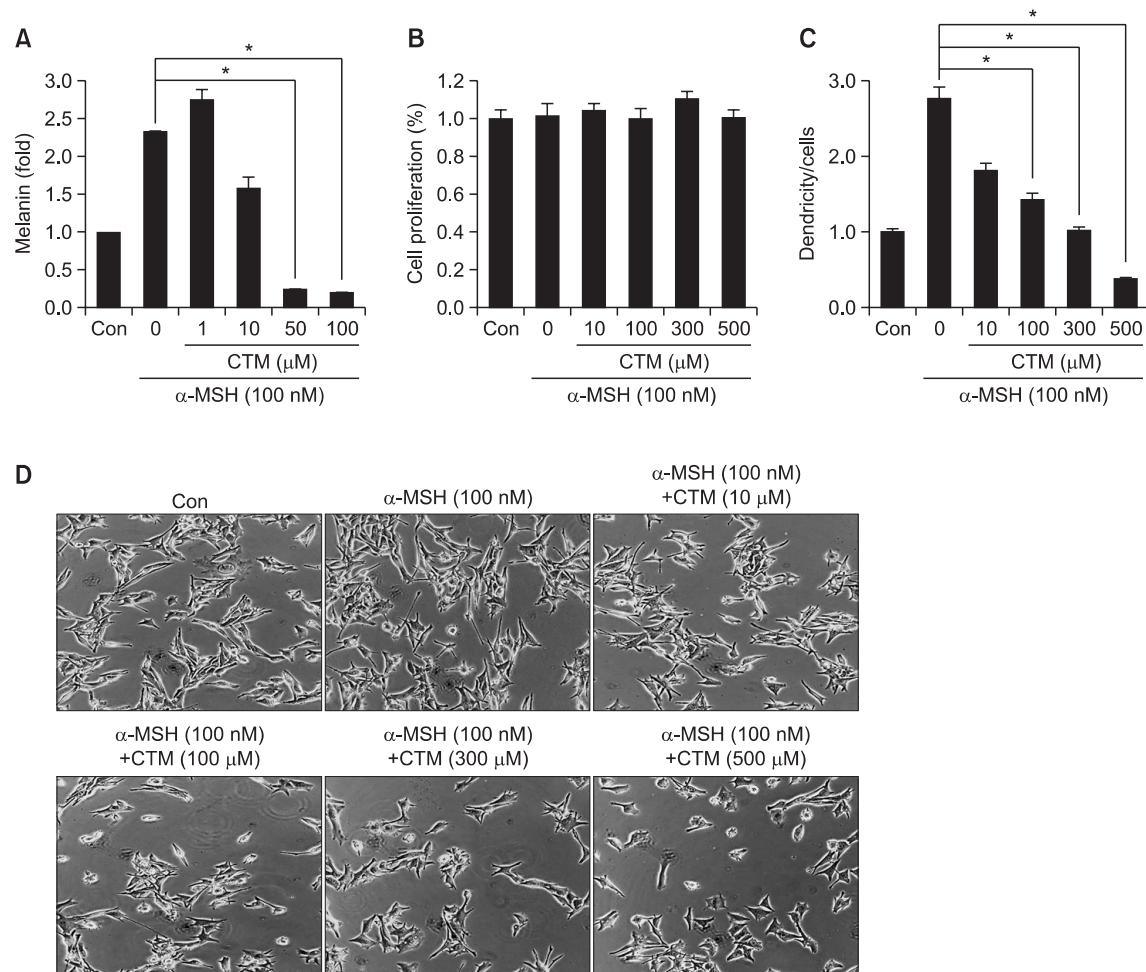


Fig. 1. Effects of CTM on pigmentation in SK-MEL-2 cells. (A) Effects of CTM on melanin production. Melanoma cells were cultured in RPMI media. The cells were seeded in a 6-well plate with or without CTM (1, 10, 50, 100 μM) and $\alpha\text{-MSH}$ (100 nM). After 2 days of incubation, the cells were washed twice with PBS, resuspended in 1 N NaOH containing 10% DMSO, and heated at 80°C for 1 hr. The absorbance of the extracted melanin was read at 405/450 nm using an ELISA microplate reader. (B) Effects of CTM on cell proliferation. SK-MEL-2 cells were cultured in a 96-well plate with or without CTM (10, 100, 300, 500 μM) and $\alpha\text{-MSH}$ (100 nM). After 48 hrs of incubation, the relative cell proliferation was determined using a Cell Counting kit-8. (C) Plot of effects of CTM on dendrite extension of SK-MEL-2 cells. (D) Photographs of effects of CTM on dendrite extension of SK-MEL-2 cells. In (C) and (D), SK-MEL-2 human melanoma cells were cultured in DMEM (2% FBS) media. The cells were seeded in a 6-well plate with or without CTM and $\alpha\text{-MSH}$. After 48h of incubation, the cells with and without “dendricity” (i.e. the appearance of cell extensions as a function of time) were counted and photographed under Zeiss microscopy (20x magnification) using a Real-time image system. In (C), the average dendrite lengths (n=20) in the melanoma cells were measured using the Image tool program. * $p < 0.05$.

RESULTS

CTM suppressed melanin production, cell proliferation and dendrite extension of SK-MEL-2 cells

To confirm CTM's antimelanogenic functionality, we examined its effect on SK-MEL-2 cell melanogenesis. Cells were incubated with the indicated concentrations of CTM for 24 hrs, and, 2 hrs before CTM treatment, melanogenesis was stimulated by $\alpha\text{-MSH}$ (100 nM). As a result of this $\alpha\text{-MSH}$ stimulation, the cells' melanin content was increased considerably (Fig. 1A), subsequent to which, CTM dose-dependently decreased it (Fig. 1A). Notably, CTM showed no cytotoxic activity in the doses used (Fig. 1B). We also examined the effects of CTM on the SK-MEL-2 cells' dendricity. Whereas $\alpha\text{-MSH}$ -induced well-developed dendricity, dendrite outgrowth was sup-

pressed by CTM (Fig. 1C, D). These results suggest that CTM suppresses $\alpha\text{-MSH}$ -stimulated melanogenesis of SK-MEL-2.

CTM suppressed $\alpha\text{-MSH}$ -induced expressions of melanogenic proteins and Tgase-2

We examined the effect of CTM on the expressions of SK-MEL-2 melanogenic proteins including TRP-1, TRP-2 and MITF by Western blotting. Treatment of the SK-MEL-2 cells with $\alpha\text{-MSH}$ resulted in increased expressions of TRP-1, TRP-2 and MITF (Fig. 2). However, pretreatment with CTM decreased their expressions. Next, because Tgase-2 expression is up-regulated during advanced stages of malignant melanomas (Fok *et al.*, 2006), we investigated whether CTM suppression of melanogenic proteins is correlated with Tgase-2 expression. Interestingly, the $\alpha\text{-MSH}$ -treated cells showed

elevated Tgase-2 expression, which was inhibited by CTM in accordance with the melanogenic proteins' expression changes (Fig. 2).

CTM reduced α -MSH-induced migration of SK-MEL-2 cells

As α -MSH is well-known to increase melanocyte migration (Brzoska *et al.*, 2008), we utilized a chemotactic migration assay to determine whether CTM, as a Tgase inhibitor, can inhibit α -MSH-induced migration of SK-MEL-2 cells. We found that α -MSH induced SK-MEL-2 cell migration and that CTM dose-dependently suppressed it (Fig. 3). These results indicated that CTM is an effective anti-migratory compound against melanoma cells.

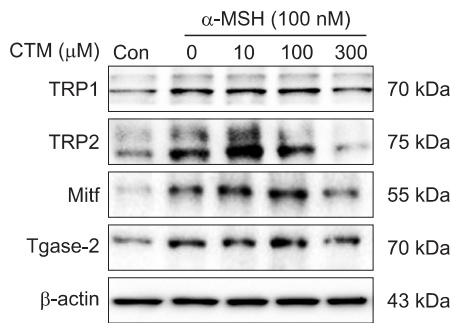


Fig. 2. Effects of CTM on MITF, TRP1, and TRP2 expressions of SK-MEL-2 cells. SK-MEL-2 cells were treated with control media or CTM (10, 100, 300 μ M) for the indicated time. Whole-cell lysates (30 μ g) were prepared, the protein level was subjected to 10% SDS-PAGE, and the expressions of several proteins were determined by Western blotting.

Tgase-2 is involved in α -MSH-induced melanogenesis of SK-MEL-2 cells

To confirm the involvement of Tgase-2 in α -MSH-induced melanogenesis of SK-MEL-2 cells, we also examined the effects of Tgase-2 gene silencing on dendrite extension and expressions of melanogenic proteins. As shown in Fig. 4A, gene silencing of Tgase-2 effectively suppressed the SK-MEL-2 cells' α -MSH-induced dendrite extension (Fig. 4A, B). Gene silencing of Tgase-2, moreover, significantly inhibited α -MSH-induced expressions of TRP-1 and TRP-2 (Fig. 4C). These results indicate Tgase-2's probable involvement in α -MSH-induced melanogenesis of SK-MEL-2 cells.

DISCUSSION

Skin pigmentation disorders including vitiligo and melasma can result from problems that arise in melanocyte proliferation, differentiation, melanogenesis, migration or dendricity (Lee *et al.*, 2012b). Pigmentation modulation is important to aesthetics and quality of life. Regardless, the involved melanogenic mechanism is still not fully understood.

Many intrinsic biological factors, including α -MSH and endothelin-1, are implicated in the pigmentation and tanning response to UV irradiation (Son *et al.*, 2014). α -MSH binding to MC1R induced the up-regulation of TYR, TRP-1, and TRP-2 via the mediation of MITF (Lee and Noh, 2013).

In the present study, we showed that α -MSH increases the production of melanin in SK-MEL-2 cells and that CTM inhibits such α -MSH-induced melanin production (Fig. 1A). CTM's anti-melanogenic activity also has been reported in other melanoma cell lines, namely MM96L and MM418c5 (Bologna *et al.*, 1995; Qiu *et al.*, 2000). CTM is believed to be reduced to cysteamine via sequestering quinines (Qiu *et al.*, 2000). Whereas such studies usually have focused on CTM's

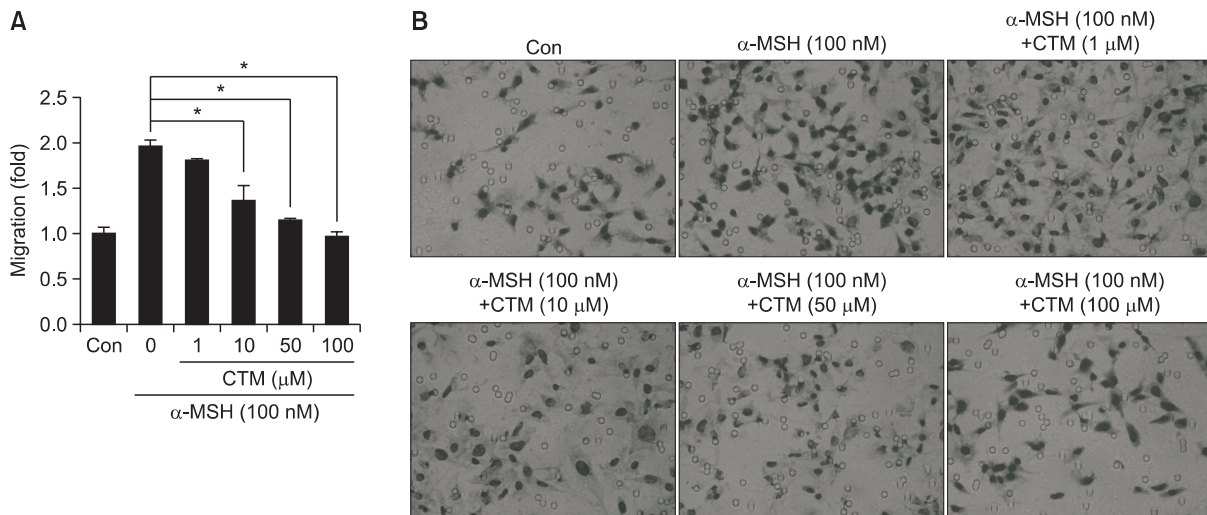


Fig. 3. Effect of CTM on chemotactic migration in SK-MEL-2 cells. (A) Plot of effect of CTM on chemotactic migration in SK-MEL-2 cells. (B) Photograph of effect of CTM on chemotactic migration in SK-MEL-2 cells. In (A) and (B), for the migration assay, the lower chamber transwells were coated with fibronectin (10 μ g/ml). SK-MEL-2 cells (5×10^4 /well) were treated with vehicle or increasing concentrations (1, 10, 50, 100 μ M) of cyst amine for 4 hrs. After incubation, the cells on the bottom side of the filter were fixed, stained by Diff-quick, and then subsequently counted under four randomly chosen high-power fields (400 \times). In (B), SK-MEL-2 cells that had migrated through the membrane after 5 hrs of incubation were stained and photographed. * $p < 0.05$.

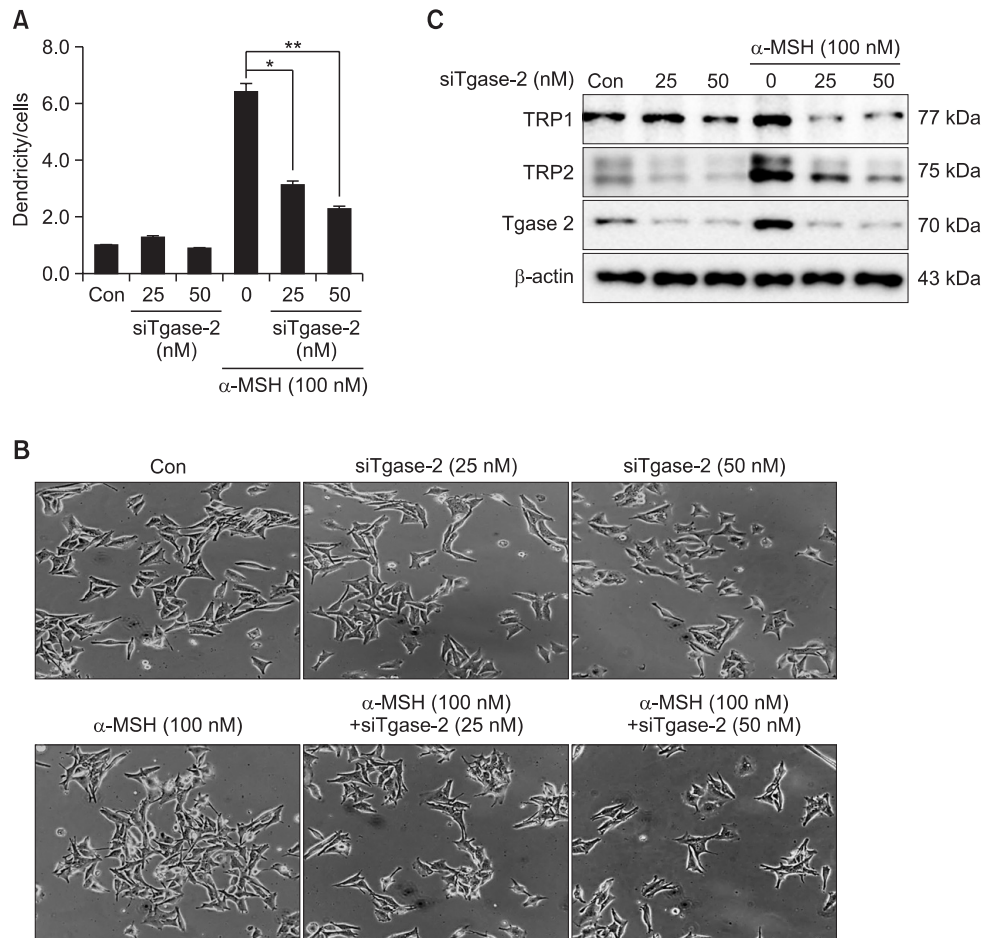


Fig. 4. Effects of Tgase2 gene silencing on dendricity and MITF, TRP1, and TRP2 expressions of SK-MEL-2 cells. (A) Effect of Tgase2 gene silencing on dendrite extension of SK-MEL-2 cells. (B) Photograph of effect of Tgase2 gene silencing on dendrite extension of SK-MEL-2 cells. (C) Effect of Tgase-2 gene silencing on MITF, TRP1, and TRP2 expressions of SK-MEL-2 cells. In (A), and (B), the SK-MEL-2 cells were transfected with siRNA of Tgase-2 for 48 hrs. After 48 hrs of incubation, the cells were treated with α -MSH. Then, the cells with and without "dendricity" (i.e. the appearance of cell extensions as a function of time) were counted and photographed under Zeiss microscopy (20 \times magnification) using a Real-time image system. In (A), the average dendrite lengths (n=20) in the melanoma cells were measured using the Image tool program. In (C), whole-cell lysates (30 μ g) were prepared, the protein level was subjected to 4-12% SDS-PAGE, and the expressions of several proteins were determined by Western blotting. * p <0.05, ** p <0.01.

involvement via the inhibition of TYR activity, we also examined the effects of CTM on expressions of melanogenesis-implicated proteins such as TRP-1, TRP-2, MITF and Tgase-2, as well as the dendricity and chemotactic migration of SK-MEL-2 cells (Fig. 2, 3). In the results, CTM at high dose did not show cytotoxic effects on SK-MEL-18 melanoma cells (Fig. 1B) (Bolognia *et al.*, 1995). α -MSH induced dendrite formation in SK-MEL-2 cells, and CTM dose-dependently inhibited it (Fig. 1C). CTM suppressed α -MSH-induced induction of TRP-1, TRP-2, MITF and Tgase-2 (Fig. 2). However, we could not observe significant effects of CTM on TYR expression (data not shown). CTM inhibited α -MSH-induced migration of SK-MEL-2 cells (Fig. 3).

CTM also is regarded as a Tgase inhibitor, though its specificity to Tgase-2 is low. Because Tgase-2 usually is involved in dendrite formation in neuronal cells (Tucholski *et al.*, 2001), and given α -MSH's induction of Tgase-2 expression (Fig. 2), we speculated that CTM's effects on α -MSH-induced melanogenesis might be attributable to Tgase-2 inhibition. Consistent

with our expectation, gene silencing of Tgase-2 suppressed α -MSH-induced dendrite formation (Fig. 4A, B). Tgase-2 gene silencing, furthermore, blocked α -MSH-induced TRP-1, TRP-2, MITF, and Tgase-2 expression (Fig. 4C). These results all pointed to Tgase-2's involvement in SK-MEL-2 cell melanogenesis, though the specific mechanism remains unclear. However, its known involvement in the differentiation of several cell types, including chondrocytes and oligodendrocytes suggests that its role in melanogenesis might be via the differentiation of melanocytes (Van Strien *et al.*, 2011; Niger *et al.*, 2013). Recently, autophagy is involved in regulation of melanogenesis and Tgase-2 is involved in regulation of autophagy (Ozpolat *et al.*, 2007; Ho and Ganesan, 2011). So Tgase-2-involvement in autophagy might be one of plausible explanations for its involvement in α -MSH-induced melanogenesis. However this speculation requires further studies. In summation, we showed both that Tgase-2 is involved in α -MSH-induced melanogenesis and that it is a promising target for its control.

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