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Loss of EMP2 Inhibits Melanogenesis of MNT1 Melanoma Cells via Regulation of TRP-2

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Abstract

Melanogenesis is the production of melanin from tyrosine by a series of enzyme-catalyzed reactions, in which tyrosinase and DOPA oxidase play key roles. The melanin content in the skin determines skin pigmentation. Abnormalities in skin pigmentation lead to various skin pigmentation disorders. Recent research has shown that the expression of EMP2 is much lower in melanoma than in normal melanocytes, but its role in melanogenesis has not yet been elucidated. Therefore, we investigated the role of EMP2 in the melanogenesis of MNT1 human melanoma cells. We examined TRP-1, TRP-2, and TYR expression levels during melanogenesis in MNT1 melanoma cells by gene silencing of EMP2. Western blot and RT-PCR results confirmed that the expression levels of TYR and TRP-2 were decreased when EMP2 expression was knocked down by EMP2 siRNA in MNT1 cells, and these changes were reversed when EMP2 was overexpressed. We verified the EMP2 gene was knocked out of the cell line (EMP2 CRISPR/Cas9) by using a CRISPR/Cas9 system and found that the expression levels of TRP-2 and TYR were significantly lower in the EMP2 CRISPR/Cas9 cell lines. Loss of EMP2 also reduced migration and invasion of MNT1 melanoma cells. In addition, the melanosome transfer from the melanocytes to keratinocytes in the EMP2 KO cells cocultured with keratinocytes was reduced compared to the cells in the control coculture group. In conclusion, these results suggest that EMP2 is involved in melanogenesis via the regulation of TRP-2 expression.

Key Words: EMP2, TYR, TRP-1, TRP-2, Melanogenesis, Melanosome transfer

INTRODUCTION

Skin pigmentation depends on the cooperation of melanocytes and keratinocytes (KC), and the delivery and distribution of melanosomes to KC is an important process in skin pigmentation (Lee, 2015; Serre *et al*., 2018). Skin pigmentation disorders can be widespread, and the resulting pigmentation changes are classified as depigmentation, hypopigmentation, and hyperpigmentation (Enkhtaivan and Lee, 2021). Skin hyperpigmentation is an especially common skin condition and includes melasma, melanoma, and post-inflammatory hyperpigmentation (Lambert *et al*., 2019). It is caused by sun exposure, hormones, skin damage, and inflammation. However, the lack of effective therapeutics available to treat skin hyperpigmentation suggests the need for further analysis of appropriate targets and various mechanisms regulating the fate of melanin, including melanogenesis and degradation.

In mammals, melanocytes regulate skin pigmentation by catalyzing melanin biosynthesis via three melanocyte-specific enzymes: tyrosinase (TYR), tyrosinase-related protein 1 (TRP-1) and tyrosine-related protein 2 (TRP-2) (Kim *et al*., 2014; Tuerxuntayi *et al*., 2014; Ito and Sato, 2021). TYR is involved

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in melanin biosynthesis as a rate-limiting enzyme, catalyzing the hydroxylation of L-tyrosine to L-3, 4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to dopaquinone is catalyzed by TYR. Once dopaquinone is oxidized to dopachrome, TRP-2 catalyzes dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (Yokoyama *et al*., 1994; Lee *et al*., 2011; Enkhtaivan and Lee, 2021). In addition, TRP-1 is involved in melanogenesis by regulating the activity of TYR (Kobayashi *et al*., 1994).

EMP2 is a member of the growth arrest-specific-3/peripheral myelin protein-22 (GAS3/PMP22) family of tetraspan proteins (Wadehra *et al*., 2002). It plays a role in the regulation of various processes, including cell migration, cell proliferation, cell contraction, cell adhesion, apoptosis, endocytosis, and cholesterol homeostasis (Wadehra *et al*., 2008; Wang *et al*., 2017). EMP2 regulates the surface expression of $\alpha5\beta3$ integrin in the endometrium and regulates adhesion by interacting with $\beta1$ integrin (Wadehra *et al*., 2002; Wang *et al*., 2017). EMP2 is also involved in the regulation of the epithelial-mesenchymal transition (EMT) process and the differentiation of cells into functional cardiomyocytes (Liu *et al*., 2019). Recent studies have shown that EMP2 is downregulated in melanoma at the protein and mRNA levels because of autophagic proteolysis mediated by the mTOR pathway (Wang *et al*., 2019).

However, there are no detailed studies of the link between EMP2 and melanogenesis as yet. In this study, we investigated the involvement of EMP2 in melanogenesis. We found that EMP2 regulates the melanogenesis of MNT1 melanoma cells via the control of TRP-2 expression.

MATERIALS AND METHODS

Materials

Minimum essential medium (MEM) was purchased from Gibco (Carlsbad, CA, USA). Lipofectamine™ 3000 reagent was purchased from Invitrogen (Carlsbad, CA, USA). Anti-EMP2 antibody and anti-tyrosinase antibody were obtained from Abcam (Cambridge, UK). Anti-TRP-1, anti-TRP-2, anti-MITF and anti-β actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alexa FluorTM 488 donkey goat anti-mouse IgG antibody and Alexa Fluor 594® goat anti-rabbit IgG antibody were supplied from Molecular Probes (Eugene, OR, USA). AccuPower® PCR PreMix was purchased from Bioneer Inc (Daegu, Korea).

Cell culture

The human melanoma (MNT-1) cells were acquired as a gift from professor Ai-Young Lee (Department of Dermatology, Dongguk University Ilsan Hospital, Goyang, Korea). MNT-1 cells were maintained in MEM (Gibco) containing 10% Dulbecco's MEM (Cytiva), 2% 1M HEPES (Sigma-Aldrich Co., St. Louis, MO, USA), 20% FBS (Hyclone, South Logan, UT, USA), streptomycin (100 μg/mL), and penicillin (100 U/mL). The cells were washed twice in Dulbecco's phosphate-buffered saline (DPBS). The cells were maintained at 37°C in a humidified atmosphere containing 5% carbon dioxide.

Gene correlation analysis using GEPIA 2

The correlation of EMP2 expression levels with TRP-1, TRP-2 or TYR expression in GTEx skin datasets was investigated using Pearson correlation statistics on the Correlation Analysis module of GEPIA 2 (GTEx Consortium, 2017; Tang *et al*., 2019).

Western blot

Western blot analysis was conducted in accordance with a previous report (Rho *et al*., 2021). Cells were washed twice with PBS and whole-cell lysates were prepared in RIPA buffer containing protease inhibitor cocktail and Xpert Phosphatase Inhibitor Cocktail Solution (GenDEPOT, Barker, TX, USA) on ice for 15 min. Cell lysates were centrifuged at 15,000 rpm for 15 min at 4°C, and the resultant supernatants were subjected to Western blotting (Rho *et al*., 2021). The total protein concentration was quantified using Coomassie brilliant blue. Equal amounts of protein were separated by electrophoresis using a 10% SDS–polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking with 3% skim milk in TBST for 1h at room temperature, the membrane was then incubated with the appropriate primary antibodies in 3% BSA at 4°C overnight. And, after Tris-buffered saline containing Tween (TBST) washing, the membrane was incubated with HRP-conjugated secondary antibody (1:5,000) at room temperature for 60 min. The proteins were detected using PowerOptiECL detection reagent (Bionote, Seoul, Korea) and exposure to X-ray film.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was carried out following the method described in a previous report (Kim *et al*., 2021a). Total RNA was isolated from cells using the Trizol reagent (Invitrogen). NanoDropTM 2000 was used to measure RNA concentrations and RNA purity. RNA was reverse transcribed into complementary DNA, which was produced from total RNA with oligo(dt) 15 primer (5 μg/μL), 10 mM dNTP mix (0.5 μM), 5×reaction buffer, 25 mM $MgCl_2$ and reverse transcriptase. The sequences of PCR primers were: forward EMP2 5′-GCATCGCCTTCTTCATCTTC -3′; reverse EMP2 5′-CCTTCTCTGGTCACGGGATA-3′; forward TRP-1 5′-ACTTTGTAACAGCACCGAGGAT-3′, reverse TRP-1 5′-ATGTCCAATAGGGGCATTTTC-3′; forward TRP-2 5′-GATCACCACACAACACTGGC -3′, reverse TRP-2 5′-AGAGTCGGATCGTCTGGTCT -3′; forward TYR 5′- TTGG-CAGATTGTCTGTAGCC-3′, reverse TYR 5′-AGGCATTGTGCATGGTGCTT -3′; forward GAPDH 5′-TGGTCTCCTCTGACTTCA-3′, reverse GAPDH 5′-CGTTGTCATACCAGGAAATG-3′. The PCR profile was 94°C for 30 s (denaturing), 56°C for 30 s (annealing) and 72°C for 30 s (elongation) for 30 cycles. The PCR products were electrophoresed on a 1.5% agarose gel and photographed under ultraviolet light.

Confocal microscopy

Confocal microscopy was conducted in accordance with a previous report (Kim *et al*., 2021b). The MNT1 cells were cultured on coverslips. The cells were fixed in 4% (w/v) paraformaldehyde (PFA) for 10 min at room temperature. The fixed cells were followed by three washes in phosphate-buffered saline (PBS) with 0.1% Tween-20. Fixed cells were permeabilized in 0.5% Triton X-100 for 10 min at room temperature, followed by three washes in PBS with 0.1% Tween-20. The next step was, blocking with 3% bovine serum albumin in PBS for 1 h, also at room temperature. After three washes, EMP2 primary antibody or TRP-1 primary antibody or TRP-2 primary antibody or MITF primary antibody were incubated with coverslips overnight at 4°C. After three washes in PBS with 0.1%

Tween 20, cells were treated with species-specific second antibodies conjugated to goat anti-mouse IgG antibody (Alexa Fluor™ 488, 1:500; Molecular Probes) and goat anti-rabbit IgG antibody (Alexa Fluor™ 594, 1:500; Molecular Probes). The MNT1 cells were then reacted with the coverslips for 1 h at room temperature, followed by four washes in PBS with 0.1% Tween-20. The final samples were mounted onto slides and visualized by confocal microscopy.

Invasion assay

Invasion assays were performed using transwells that were coated with Matrigel (0.5 μg/mL) (Neuro Probe Inc., Gaithersburg, MD) following a previous report (Choi *et al*., 2020). Coat Matrigel (10 μL/well) in the transwell insert, overnight at room temperature. Trypsinized cells were suspended in a serum-free medium, and 2×10^6 cells were added to the upper chamber of the transwell inserts. Medium with 20% FBS was added to the lower chamber. After 48 h incubation with MNT1 cells, the non-invaded cells on the upper surface of the membrane were removed, the invaded cells on the lower surface of the membrane were fixed with cold methanol. And then transwell membranes were stained with hematoxylin and eosin. The cells were photographed (200× magnification) and cells in 3 randomly selected fields were counted. All experiments were repeated at least three times with two replicates each.

Migration assay

Cell migration was also studied using transwell inserts (Neuro Probe Inc., Gaithersburg, MD, USA) coated with 10 μg/mL fibronectin as described previously (Lee *et al*., 2020). Trypsinised cells were suspended in a serum-free medium, and 2×10^6 cells were added to the upper chamber of the transwell inserts. Medium with 3% FBS was added to the lower chamber incubate for 10 min. After 37°C incubation for 24 h, non-migrated cells on the upper surface of the membrane were removed. Transwell membranes were fixed and stained by Diff-quick®. The cells were photographed (200× magnification) and cells in 3 randomly selected fields were counted. All experiments were repeated at least three times with two replicates each.

Construction of MNT1 EMP2 CRISPR/Cas9 cell lines

EMP2 CRISPR/Cas9 KO plasmid and HDR plasmid were purchased from Santa Cruz Biotechnology. For CRISPR/Cas9 KO experiments, 5×10^5 MNT1 cells seed in 6 well plates in 3 mL antibiotic-free growth medium per well until they were grown 60% confluency (Lin *et al*., 2021). MNT1 cells were co-transfected with CRISPR/Cas9 KO plasmid and EMP2 HDR plasmid according to the manufacturer's instructions. After cells were co-transfected with CRISPR/Cas9 KO plasmid and HDR plasmid, cells were selected with media containing 5 μg/mL puromycin dihydrochloride (Macrogen, Seoul, Korea). And then selected cells were used for Western blot.

Melanosome transfer

Melanosome transfer was conducted in accordance with a previous report (Kim *et al*., 2017). MNT1 melanoma cells 3×10^4 were seeded on coverslips on 12 well plates. After 24 h, HaCaT (Human keratinocyte cells) cells were added when changing the 1:1 minimum essential medium/RPMI medium. Cells were co-cultured for 48 h. Co-cultures were washed phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 min at room temperature. The fixed cells were followed by three washes in phosphate-buffered saline (PBS) with 0.1% Tween-20. Fixed cells were permeabilized in 0.5% Triton X-100 for 10 min at room temperature, followed by three washes in PBS with 0.1% Tween-20. The next step was, blocking with 3% bovine serum albumin in PBS for 1 h, also at room temperature. After three washes, EMP2 primary antibody or PMEL primary anti-body were incubated with coverslips overnight at 4°C. After three washes in PBS with 0.1% Tween 20, cells were treated with species-specific second antibodies conjugated to donkey anti-rabbit IgG antibody (Alexa Fluor™ 488, 1:500; Molecular Probes) and goat anti-rabbit IgG antibody (Alexa Fluor™ 594, 1:500; Molecular Probes). Co-cultured cells were then reacted with the coverslips for 1 h at room temperature, followed by four washes in PBS with 0.1% Tween-20. The final samples were mounted onto slides and visualized by confocal microscopy.

Statistical analysis

Data are presented as mean ± standard error of the mean (SD) for at least three independent experiments performed in triplicate. Statistical analysis involved the paired Student's t-test. Differences were considered statistically significant at a level of $p<0.05$.

RESULTS

EMP2 expression is correlated with melanogenic enzyme activity

In the A375 human melanoma cell line, EMP2 acts as a tumour suppressor by inducing apoptosis (Ahmat Amin *et al*., 2019). However, the effect of EMP2 on melanogenesis has not yet been investigated. Therefore, we investigated the relationship between EMP2 and the melanogenesis-related genes TYR, TRP-1, and TRP-2 using bioinformatics analysis.

Using the correlation analysis module of GEPIA 2, we investigated the correlation of EMP2 expression with that of TRP-1, TRP-2 and TYR in the GTEx skin dataset (GTEx Consortium, 2017). In sun-exposed skin, EMP2 expression was positively correlated with TYR, TRP-1, and TRP-2 expression (R=0.3, p=0.0; R=0.23, p=0.0; R=0.24, p=0.0) (Fig. 1A left, 1B left, 1C left). It showed a significant positive correlation value (R=0.3, p=0.0) with TYR (Fig. 1A, left). However, in normal skin not exposed to the sun, the expression of TYR and TRP-1 had a decreased correlation with the expression of EMP2 (R=0.22; p=0.00056; R=0.17, p=0.00096; R=0.24, p=0.0003) (Fig. 1A right, 1B right, 1C right). These results suggest that the expression of EMP2 is correlated to the expression of melanogenesis-related players such as TYR, TRP-1, and TRP-2.

Loss of EMP2 suppresses melanogenesis and invasion and migration of MNT1 melanoma cells

To determine whether EMP2 affects melanogenesis, we investigated the effect of EMP2 gene silencing on TYR, TRP-1, and TRP-2 expressions in melanogenesis-prone MNT1 cells. When EMP2 expression was suppressed in MNT1 melanoma cells, TYR and TRP-2 were significantly down-regulated at both the protein and mRNA levels, but the expression of TRP-1 was not affected (Fig. 2A, 2B). We also performed confocal microscopy of EMP2, TRP-1, TRP-2, and MITF in MNT1 melanoma cells. EMP2 loss decreased TRP-2 expression but had little effect on MITF expression (Fig. 2C).

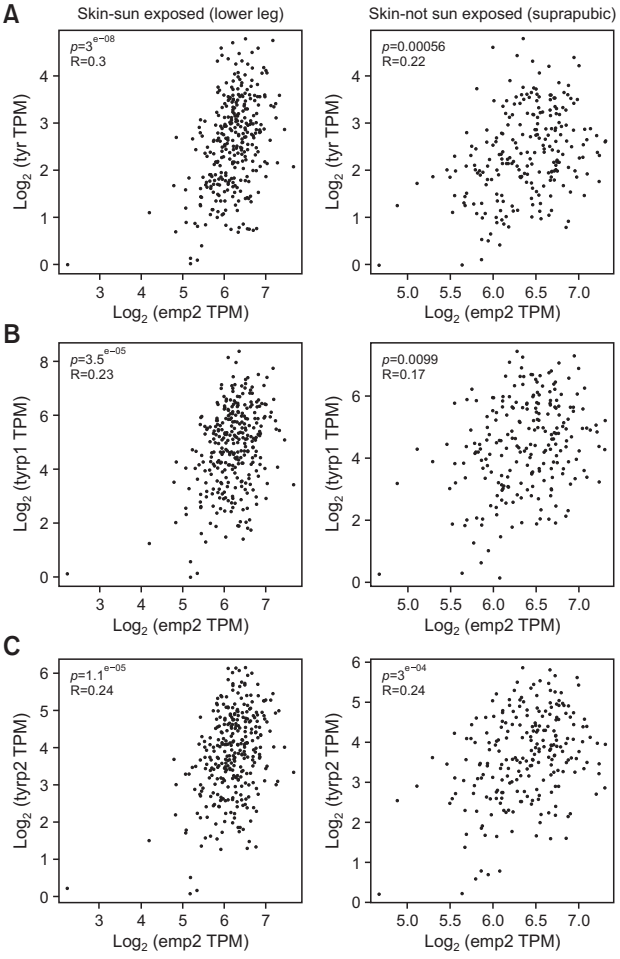

Fig. 1. The relationship of EMP2 expression levels with TRP-1, TRP-2, and TYR expressions in the GTEx skin dataset analyzed in GEPIA. The correlation of EMP2 expression levels with TRP-1, TRP-2 (DCT), and TYR expressions in the GTEx skin dataset was analyzed by Pearson's correlation coefficient. Abbreviations: GEPIA, Gene Expression Profiling Interactive Analysis; GTEx, Genotype-Tissue expression; TPM, transcripts per kilobase million (A), TRP-1 (B), TRP-2 (C), TYR.

The migration of melanocytes to areas where melanocytes are scarce is also an important process in melanogenesis (Jeon *et al*., 2007; Lee, 2015; Enkhtaivan and Lee, 2021). Therefore, we investigated the effect of EMP2 on cell invasion/migration using the Matrigel-coated assay and the transwell assay. Our results showed that invasion and migration were reduced in MNT1 cells with reduced EMP2 expression by EMP2 siRNA compared with control cells (Fig. 2D, 2E).

Overexpression of EMP2 enhances the melanogenesis, invasion, and migration of MNT1 melanoma cells

EMP2 overexpression increased the protein and mRNA expression of key enzymes regulating pigment production, such as TRP-2 and TYR, in MNT1 cells but had little effect on TRP-1 expression (Fig. 3A, 3B). We also performed confocal microscopy analysis of EMP2, TRP-1, TRP-2 and MITF in MNT1 melanoma cells. We found that overexpression of EMP2 increased TRP-2 expression but had little effect on TRP-1 and MITF expressions (Fig. 3C). We then sought to determine whether overexpression of EMP2 affects MNT1 melanoma cell migration and invasion. We determined the effect of EMP2 overexpression on cell invasion/migration using the Matrigel-coated and transwell assays. Our results indicated that invasion and migration of MNT1 cells transfected with the EMP2 plasmid were increased compared to control cells. (Fig. 3D, 3E).

EMP2 KO suppresses melanogenesis and invasion and migration of MNT1 melanoma cells and melanosome transfer of coculture of MNT1 melanoma cells with HaCaT cells

In recent years, CRISPR gene repair technology has become the most powerful approach to generating genetic models for both basic and preclinical research (Doudna and Charpentier, 2014; Lin *et al*., 2021). CRISPR/Cas9 is a targeted genome-editing tool. In this study, we used the CRISPR/Cas9 system to create a stable cell line lacking EMP2 to confirm the above results (Fig. 4A, top). It was found that the amount of melanin pigment in the cell line lacking EMP2 was noticeably lower (Fig. 4A, bottom). In the EMP2 knockout cell line, the protein and mRNA expressions of TRP-2 and TYR were significantly decreased compared to the control cells (Fig. 4B, 4C). However, there was no significant effect on TRP-1 expression. We conducted the same cell invasion and migration assays to determine the effect of EMP2 knocking out on cell invasion and migration. Our results indicated that the invasion and migration of MNT1 cells co-transfected with the EMP2 CRISPR/Cas9 and EMP2 HDR plasmids were lower than those of the control cells (Fig. 4D, 4E).

Melanin pigment is synthesized in melanocytes and accumulates in organelles called melanosomes. These melanosomes travel to the dendrites of the melanocytes and are delivered to nearby KC (Lee, 2015; Enkhtaivan and Lee, 2021). To determine whether the lack of EMP2 affects the transport of KC in the melanosome, MNT1 cells and HaCaT cells (KC) were cocultured to investigate whether the expression of PMEL, a marker of melanosome, was observed in KC under a confocal microscope. As a result, melanosome transfer from wild-type MNT cells to HaCaT cells was confirmed in control cells, whereas melanosome transfer was significantly reduced in the coculture lacking EMP2 MNT cells (Fig. 4F).

DISCUSSION

The skin is an important organ that protects against mechanical shock and pressure, temperature changes, microorganisms, radioactive substances, and chemicals (Chuong *et al*., 2002; Tobin, 2006). While performing these protective functions, skin damage, darkening, excessive pigmentation, and even skin cancer can occur in the skin. Skin colour is determined by a pigment called melanin, which is produced by melanocytes (Costin and Hearing, 2007). However, exposure to UVA/UVB, chemicals, and radioactive substances over-activates melanin production, which can lead to various skin hyperpigmentation diseases, such as melasma and post-inflammatory pigmentation (Lee, 2015; Enkhtaivan and Lee, 2021). For the treatment of these skin pigment diseases, research on the process of pigmentation and related genes and enzymes is required continuously.

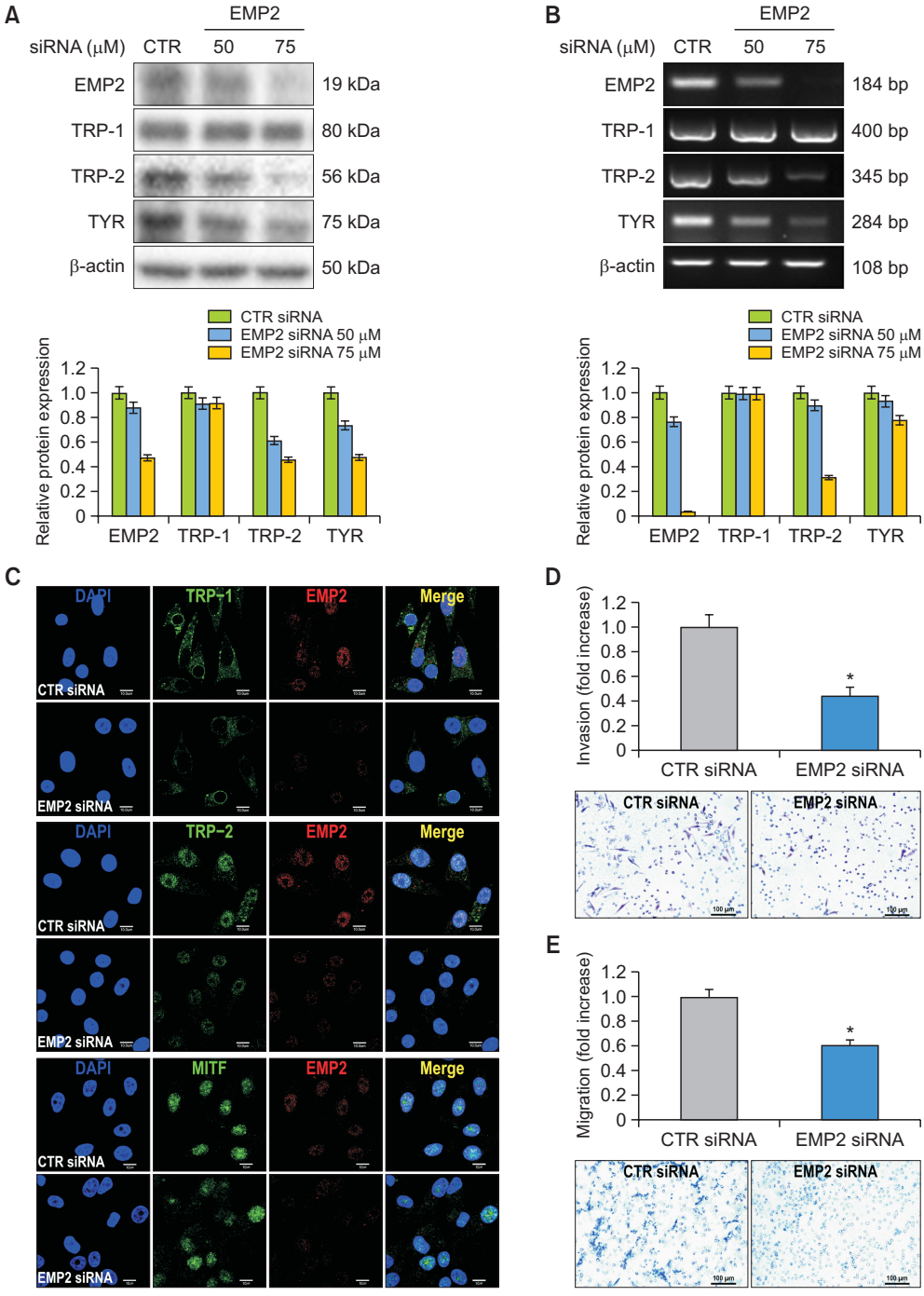

Fig. 2. Loss of EMP2 suppresses TRP-2 and TYR expression levels and invasion and migration of MNT1 melanoma cells. MNT1 cells were transfected with negative control siRNA and EMP2-specific siRNA. (A, top) The protein expressions of EMP2, TRP-1, TRP-2, and TYR were analyzed using Western blotting with β-actin as a loading control. (A, bottom) Densitometric analysis of EMP2, TRP-1, TRP-2, and TYR from Western blots. (B, top) EMP2, TRP-1, TRP-2, and TYR mRNA transcript levels were analyzed by RT-PCR, with GAPDH as a loading control. (B, bottom) Densitometric analysis of EMP2, TRP-1, TRP-2, and TYR from RT-PCR. (C) The localization of EMP2, TRP-1, TRP-2, and MITF. Immunostaining was performed using MITF, TRP-1, TRP-2 (green), EMP2 (red), and DAPI (blue). (D) MNT1 melanoma cells were transfected with EMP2-specific siRNA for 48 h, and lower chamber transwells were coated with Matrigel (0.5 μg/mL). (D, top) Loss of EMP2 reduced MNT1 melanoma cell invasion. (D, bottom) Images of invaded MNT1 melanoma cells. (E) MNT1 melanoma cells were transfected with EMP2-specific siRNA for 48 h and allowed to migrate through fibronectin (10 μg/mL)-coated membrane in transwells. (E, top) Loss of EMP2 reduced MNT1 melanoma cell migration. (E, bottom) Images of migrated MNT1 melanoma cells. A *p value<0.05 was considered significant and error bars, ± SD.

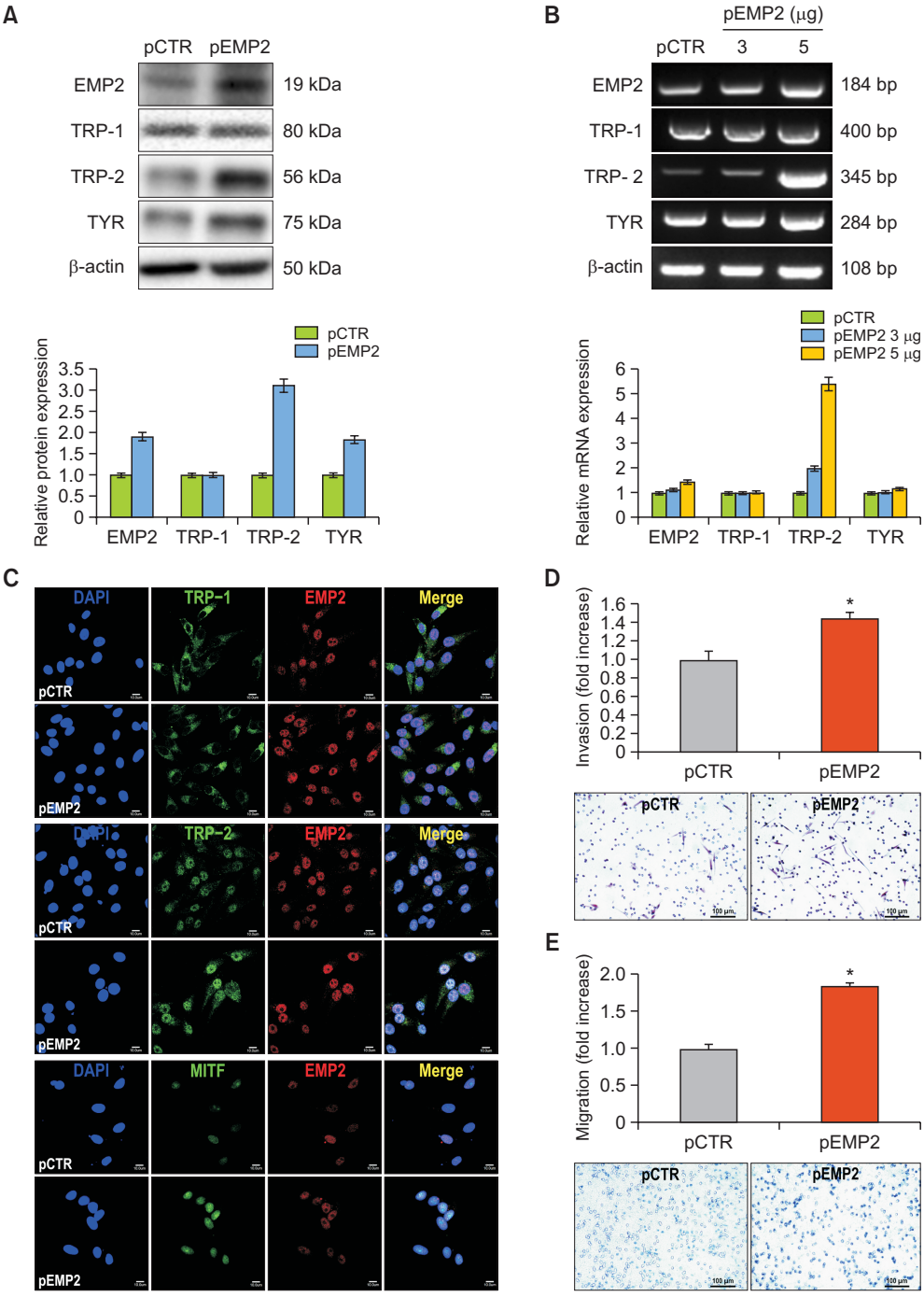

Fig. 3. Overexpression of EMP2 enhances TRP-2 and TYR expression levels and invasion and migration of MNT1 melanoma cells. MNT1 cells were transfected with a plasmid containing EMP2 and control plasmid DNA. (A, top) EMP2, TRP-1, TRP-2, and TYR protein expression levels were analyzed using Western blotting, with β-actin as a loading control. (A, bottom) Densitometric analysis of EMP2, TRP-1, TRP-2, and TYR from Western blots. (B, top) EMP2, TRP-1, TRP-2, and TYR mRNA levels were analyzed using RT-PCR, with GAPDH as a loading control. (B, bottom) Densitometric analysis of EMP2, TRP-1, TRP-2, and TYR from RT-PCR. (C) The localization of EMP2, TRP-1, TRP-2, and MITF. Immunostaining was performed using MITF, TRP-1, TRP-2 (green), EMP2 (red), and DAPI (blue). (D) MNT1 melanoma cells were transfected with EMP2 plasmid DNA for 48 h, and lower chamber transwells were coated with Matrigel (0.5 μg/mL). (D, top) Overexpressed EMP2 enhanced MNT1 melanoma cell invasion. (D, bottom) Images of invaded MNT1 melanoma cells. (E) MNT1 melanoma cells were transfected with EMP2 plasmid DNA for 48 h and allowed to migrate through fibronectin (10 μg/mL)-coated membrane in transwells. (E, top) Overexpressed EMP2 enhanced MNT1 melanoma cell migration. (E, bottom) Images of migrated MNT1 melanoma cells. A **p* value<0.05 was considered significant and error bars, ± SD.

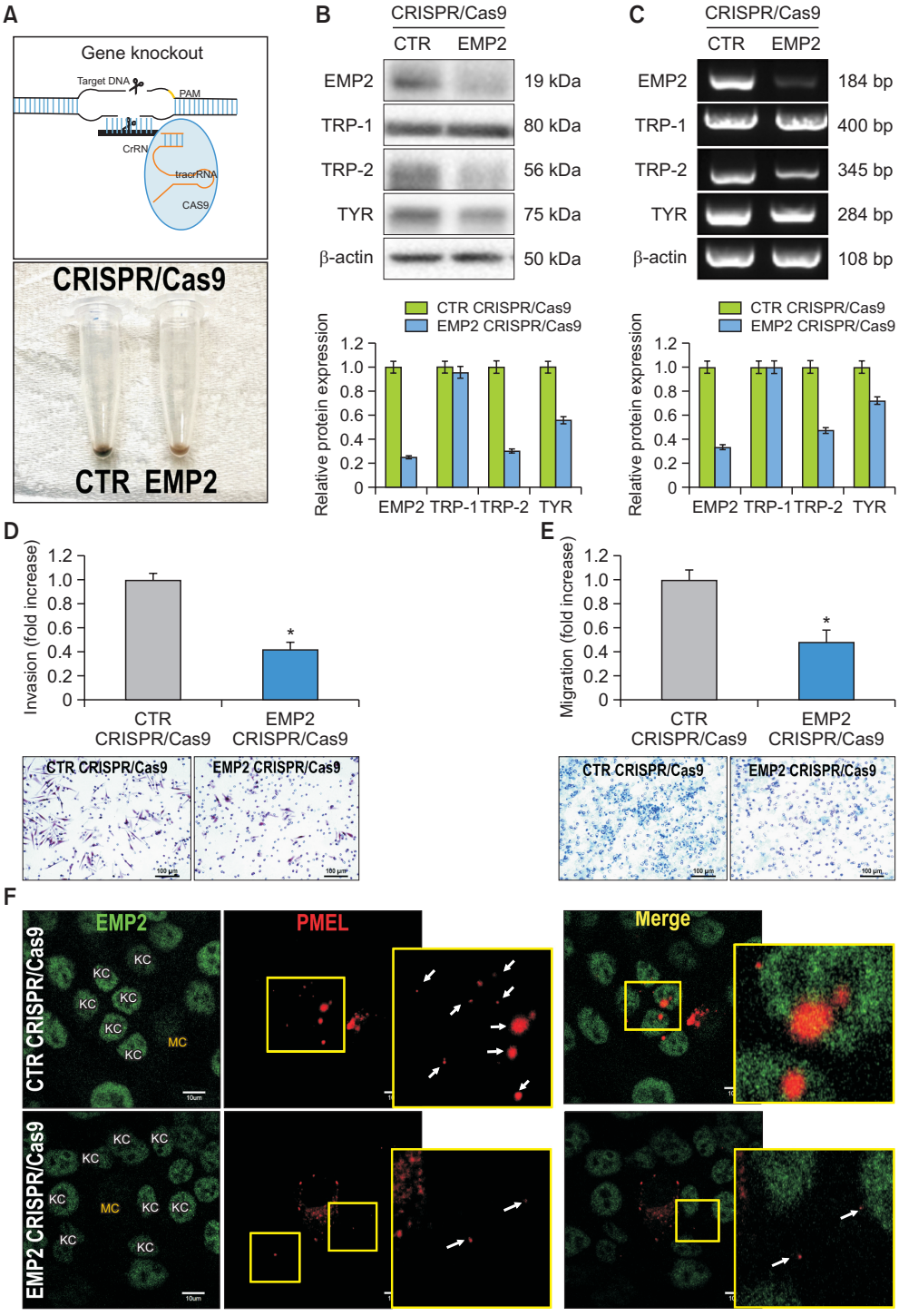

Fig. 4. EMP2 KO decreases expressions of TRP-2 and TYR in MNT1 melanoma cells and melanosome transfer in coculture of MNT1 and HaCaT cells. (A, top) Scheme of EMP2 KO by CRISPR/Cas9. (A, bottom) MNT1 cells were co-transfected with the control CRISPR/Cas9 plasmid and EMP2 CRISPR/Cas9 plasmid/HDR plasmid. Images of the effect of EMP2 gene KO in MNT1 cells. (B, top) EMP2, TRP-1, TRP-2, and TYR protein expressions were analyzed using Western blotting, with β-actin as a loading control. (B, bottom) Densitometric analysis of EMP2, TRP-1, TRP-2, and TYR from Western blots. (C, top) EMP2, TRP-1, TRP-2, and TYR mRNA expression levels were analyzed using RT-PCR, with GAPDH as a loading control. (C, bottom) Densitometric analysis of EMP2, TRP-1, TRP-2, and TYR from RT-PCR. (D, E) EMP2 KO reduces the invasion and migration of MNT1 melanoma cells. (D) MNT1 melanoma cells were co-transfected with EMP2 CRISPR/Cas9 plasmid and HDR plasmid, and lower chamber transwells were coated with Matrigel (0.5 μg/mL). (D, top) EMP2 KO reduced MNT1 melanoma cell invasion. (D, bottom) Images of invaded MNT1 melanoma cells. (E) MNT1 melanoma cells were co-transfected with specific CRISPR/Cas9 plasmid and HDR plasmid targeting EMP2 and allowed to migrate through fibronectin (10 μg/mL)-coated membrane in transwells. (E, top) EMP2 KO reduced MNT1 melanoma cell migration. (E, bottom) Images of migrated MNT1 melanoma cells. (F) Confocal microscopic examination of PMEL and EMP2 in MNT1/HaCaT coculture cells. Double immunolabeling of MC/KC cocultures with PMEL antibody (red) and anti-EMP2 antibody (green). KC: keratinocyte, MC: melanocyte, PMEL: melanosome marker. A *p value<0.05 was considered significant and error bars, ± SD.

EMP2 is a cell membrane protein and is known as a cancer biomarker and therapeutic target. It functions as an oncogene in brain cancer and endometrial cancer and as a tumour suppressor gene in B cell lymphoma, lung cancer, melanoma, and bladder cancer (Wang *et al*., 2001; Qin *et al*., 2014; Li *et al*., 2015; Lee *et al*., 2016; Kiyohara *et al*., 2017; Wang *et al*., 2019; Li *et al*., 2021; Ma *et al*., 2021). EMP2 has been identified as a novel melanoma suppressor regulated by mTOR-mediated autophagy (Wang *et al*., 2019). However, there are no detailed studies of the link between EMP2 and skin pigmentation. Using the GEPIA2 tool (Fig. 1), we found that EMP2 is associated with two melanogenesis-related enzymes TYR and TRP-2. Therefore, we conducted a study on the role of EMP2 in melanogenesis.

Our results revealed a role for EMP2 in the pigmentation of MNT1 melanoma cells. EMP2 deficiency reduced the expression of the major melanin biosynthetic enzymes TRP-2 and TYR (Fig. 2, 4). Conversely, overexpression of EMP2 increased the expression levels of TYR and TRP-2 (Fig. 3), and this effect was most remarkable for TRP-2. This strong effect of EMP2 on TRP-2 expression may be associated with the fact that TRP-2 and TRP-1 are trafficked by distinct pathways in B16 melanoma cells (Negroiu *et al*., 2005). Thus, it seems highly likely that EMP2 is involved in an early secretory pathway that includes TRP-2 as cargo but not TRP-1. However, more research is required to confirm this proposition (Negroiu *et al*., 2005). Interestingly, EMP2, present in the perinuclear region, is also known to be present in the Golgi region (Wadehra *et al*., 2004). As a result of confirming the locations and expressions of EMP2, TRP-1, TRP-2, and MITF using confocal microscopy, it was found that the expression of TRP-2 was lower (Fig. 3E). These data confirm that loss of EMP2 reduces melanogenesis, but more research is needed to decipher the mechanisms.

EMP2 depletion decreased MNT1 cell invasion and migration, and increased EMP2 expression increased cell invasion and migration (Fig. 2D, 2E, 3D, 3E, 4D, 4E). The report that the loss of EMP2 reduces cell migration is consistent with the inhibition of neutrophil transendothelial migration in EMP2$^{-/-}$ mice (Lin *et al*., 2020). Moreover, an increase in EMP2 expression is consistent with previous findings that it also increases the migration of glioblastoma cells. However, the results conflict with studies that loss of EMP2 in lung cancer cell lines increases cell migration and invasion (Lee *et al*., 2016; Ma *et al*., 2021). This difference seems to be related to the cell context or the microenvironment surrounding the cells.

Melasma is hypermelanosis required for sun-exposed skin and is increased by 50-70% of pregnant women and menstruation (Bolanca *et al*., 2008; Lee, 2015; Mobasher *et al*., 2020). Estrogens, particularly sex hormones, such as 17β-estradiol (E2) and progesterone, are factors involved in the regulation of pigmentation (Natale *et al*., 2016). Estradiol levels are higher in the serum of women with melasma than in control women without melasma (Hassan *et al*., 1998). Interestingly, EMP2 expression is increased by estrogen and progesterone (Wadehra *et al*., 2008). Therefore, the involvement of EMP2 in melanogenesis may partially explain the increase in freckles caused by hormones, such as estrogen, during pregnancy. Conversely, if EMP2 expression is increased, it seems that symptoms can be improved by increasing melanogenesis in skin diseases caused by pigment deficiency, such as vitiligo. In conclusion, EMP2 represents a potential therapeutic target for the treatment of various skin pigmentation diseases.

CONFLICT OF INTEREST

The authors have declared that no competing interest exists.

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