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Inhibitory effects on melanogenesis by thymoquinone are mediated through the β-catenin pathway in B16F10 mouse melanoma cells

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Abstract. Thymoquinone (TQ) is a component found in the seeds of *Nigella sativa*, an annual plant growing on the Mediterranean coast, and is known for its anticancer and anti-inflammatory effects. However, to date, at least to the best of our knowledge, limited studies are available examining the molecular mechanisms through which TQ inhibits melanogenesis. Accordingly, this study aimed to treat B16F10 mouse melanoma cells with TQ to investigate its apparent effects and its molecular regulatory mechanisms. Treatment of the B16F10 cells with 10, 15 and 20 μM of TQ for 48 h resulted in a dose-dependent decrease in the expression of microphthalmia-associated transcription factor (MITF), tyrosinase expression and tyrosinase activity, and these treatments simultaneously led to a decrease in the protein expression and transcription of β-catenin, a Wnt signaling pathway protein. Pre-treatment of the cells with the proteasome inhibitor, MG132, to confirm the inhibition of melanogenesis through the β-catenin pathway by TQ treatment resulted in an increase in the expression of β-catenin that was initially reduced by TQ, and the expression and activity of MITF and tyrosinase also increased. Pre-treatment with LiCl, which is known to inactivate glycogen synthase kinase 3β (GSK3β) by inducing the phosphorylation of the Ser-9 site, resulted in an increased phospho-GSK3β expression accompanied by β-catenin that was initially reduced by TQ, and the recovery of the expression and activity of tyrosinase was also confirmed. The transfection of S37A cDNA into B16F10 cells that overexpress β-catenin resulted in the recovery of β-catenin expression that was initially reduced by TQ, and this treatment also recovered the expression and activity of tyrosinase. When zebrafish eggs were treated with 1, 2.5 and 5 μM of TQ at 10 h following fertilization, their melanin content decreased in a dose-dependent manner. On the whole, these findings demonstrated that the inhibition of melanogenesis in B16F10 mouse melanoma cells by TQ treatment resulted from the inhibition of the β-catenin pathway and confirmed that TQ treatment inhibited melanogenesis in zebrafish.

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Abbreviations: TQ, thymoquinone; PTU, phenylthiourea; MITF, microphthalmia-associated transcription factor; GSK3, glycogen synthase kinase 3; α-MSH, α-melanocyte stimulating hormone; PMSF, phenylmethylsulfonyl fluoride

Key words: thymoquinone, melanogenesis, β-catenin, B16F10, zebrafish

Introduction

Melanin, which is found in various animal tissues, such as the skin, eyes and hair, is a substance that determines skin or hair colour and protects the skin from ultraviolet rays by contributing to the reduction of cell damage by eliminating reactive oxygen species (ROS) generated within the skin (1). Melanogenesis occurs in specialized organelles, such as the melanosome of melanocytes, and can be prompted a variety of paracrine cytokines, including α-melanocyte stimulating hormone (α-MSH) (2,3). All these factors activate the expression and activation of pigment-related proteins, such as microphthalmia-associated transcription factor (MITF), tyrosinase (TYR), tyrosine-related protein-1 (TRP-1) and tyrosine-related protein-2 (TRP-2) (4). All signal transduction pathways include MITF, the master regulator of melanogenesis, which upregulates the expression of the melanogenesis-related enzymes, TYR, TRP-1 and TRP-2 (5,6). Skin structure consists of the epidermis, dermis and subcutaneous fat. In the epidermis, keratinocytes are present in the spinous layer, and melanocytes are present in the basal layer. Each melanocyte is surrounded by approximately 10-40 keratinocytes (7). Melanogenesis refers to a series of processes through which melanin is synthesized. When thymidine dinucleotides (pTT), indicative of DNA damage in keratinocytes found in the spinous layer caused by UV rays, are induced, p53, a tumor suppressor protein, is activated and melanogenesis is increased (8). Within keratinocytes, activated p53 stimulates the promoter of proopiomelanocortin (POMC), a precursor of

α-MSH. α-MSH binds to the melanocortin 1 receptor (MC1R) within melanocytes to activate adenylate cyclase through a corresponding G-protein, ultimately resulting in increases in the level of intracellular cAMP and the activation of protein kinase A (PKA). PKA activates cAMP response element binding protein (CREB) by phosphorylation, and activated CREB induces the transcription of MITF (9,10). Tyrosinase transcribed by MITF is a membrane protein that fuses with melanosomes derived from lysosomes to oxidize L-tyrosine and L-DOPA, and synthesize melanin through a series of processes (9). Tyrosinase, a di-copper metalloprotein (11), is an essential element in melanogenesis that acts as a rate limiting enzyme. The glycosylation of tyrosinase affects the activity of tyrosinase (12). Melanosomes undergo a 4-stage maturation process and migrate to dendrites through microtubules, after which they are relayed to keratinocytes by various mechanisms such as exocytosis, cytophagocytosis and the fusion of plasma membrane vesicles to ultimately help prevent DNA damage from UV radiation (13). Malignant melanoma is a type of cancer that develops in melanocytes, and UV exposure is the major cause of this malignancy among individuals possessing low levels of skin pigment (14).

β-catenin is encoded by the CTNNB1 gene (15), and phosphorylation facilitated by glycogen synthase kinase 3 (GSK3) and casein kinase (CK-1) that form a complex with adenomatous polyposis coli (APC) and axin protein (16). The GSK3β-induced phosphorylation of the Ser-33 and 37 sites of β-catenin (16,17) is essential for recognition by the β-TrCP E3 ubiquitin ligase (18). β-catenin conjugated to ubiquitin delivered by the E3 ubiquitin ligase is degraded by the proteasome (17). The presence of the cAMP and Wnt/β-catenin pathways in the upstream pathway of MITF was confirmed through previous studies. The MITF promoter closest to the common downstream exon is known as the M promoter and appears to be selectively expressed in melanocytes (19). Wnt/β-catenin signal transduction is important for the differentiation of melanocytes from neural ridges (20). The binding of the WNT protein to the Frizzled receptor causes the interaction of β-catenin with the LEF/TCF transcription factor, leading to the MITF-M promoter (21). In addition, transcription factors involved in the regulation of the MITF-M promoter include paired box gene 3 (PAX3), cAMP-reactive element binding protein (CREB), SRY (sex determination region Y)-box 10 (SOX10), lymphoid enhancer-binding factor (LEF, also known as TCF), one cut domain 2 (ONECUT-2) and MITF itself (22,23). In this study, the prevention of melanin production by thymoquinone (TQ) was principally investigated by WNT/β-catenin signaling.

Currently, a number of reagents are known to supresses melanogenesis by inhibiting mRNA transcription, interfering with glycosylation, inhibiting catalytic ability and promoting the degradation of tyrosinase (24). Among these, albutin, which is well known as a whitening agent, is an inhibitor that acts competitively with the substrate L-tyrosine without affecting the transcription of tyrosinase (25). Kojic acid chelates copper, which is required in the active sites of tyrosinase (26). Phenylthiourea (PTU) induces the degradation of tyrosinase at the post-translational level and also inhibits its catalytic activity (27,28). It has been suggested, however, that the use of these reagents as whitening or therapeutic agents for pigmented lesions caused by the excessive secretion of melanin pigment may be harmful to the skin. Consequently, the need to develop ingredients containing natural extracts is increasing.

TQ is a phytochemical that is a component found in the seeds of *Nigella sativa*, an annual plant that grows on the Mediterranean coast. Seeds from *Nigella sativa* containing TQ, a natural therapeutic agent, are processed in oil and are sold as a health supplement. TQ has been shown to induce the apoptosis of human colorectal cancer cells through a p53-dependent mechanism (29), and has also been shown to exert anti-inflammatory effects on pancreatic cancer cells (30), and to exert liver protective effects on isolated rat liver cells (30). Recently, TQ has been extensively studied in the context of both basic and clinical research as a therapeutic agent for cancer treatment (31,32). Various biological effects of TQ, including its anticancer, anti-inflammatory (33) and antioxidant effects, have been examined. However, to date, at least to the best of our knowledge, studies on the molecular mechanisms through which TQ inhibits melanogenesis are limited.

Therefore, the present study treated B16F10 mouse melanoma cells with TQ and investigated the inhibition of melanogenesis and the molecular mechanisms underlying this inhibition. Treatment of the B16F10 cells with TQ resulted in a dose-dependent decrease in the expression of MITF and tyrosinase that was accompanied by decreased tyrosinase activity. These treatments also simultaneously led to a decrease in the protein expression and transcription of β-catenin, a Wnt signaling pathway protein. Pre-treatment with MG132, a proteasome inhibitor, to examine the inhibition of melanogenesis through the β-catenin pathway by TQ treatment, revealed an increase in the expression of β-catenin that was initially reduced by TQ, and the expression and activity of MITF and tyrosinase was also increased. Pre-treatment with LiCl, which is known to inactivate GSK3β by inducing the phosphorylation of the Ser-9 site, resulted in an increased phospho-GSK3β expression accompanied by β-catenin that was initially reduced by TQ, and the recovery of the expression and activity of tyrosinase was also confirmed. Transfection of S37A cDNA into B16F10 cells that overexpress β-catenin resulted in the recovery of β-catenin that was initially reduced by TQ, and this treatment also recovered the expression and activity of tyrosinase. Additionally, zebrafish were used in an animal experiment to investigate the inhibition of melanogenesis by TQ *in vivo*.

In this study, we aimed to determine whether TQ may be used as a therapeutic potential compound due to its anti-proliferative and anti-melanogenic effects by the inhibition of the β-catenin pathway.

Materials and methods

Cells and cell culture. B16F10 mouse melanoma cells used in this study were obtained from the Korean Cell Line Bank. The cells were added to Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS, WELGENE), 50 μg/ml of streptomycin (Sigma-Aldrich) and 50 U/ml of penicillin (Sigma-Aldrich) and incubated in 5% CO_2, 37˚C

incubator. The cells were subcultured in a culture dish at a density of $2x10^5$ cell/dish. The medium was exchanged every 2-3 days, and when the cell density in the culture dish reached 60%, the cells were treated with the reagents (TQ; Sigma-Aldrich; 274666, PTU; Sigma-Aldrich; P7629, MG132; Sigma-Aldrich; C221, LiCl; Calbiochem; 274666, NaCl; SAMCHUN; S0484, KCl; Duchefa biochemie; 7447-40-7) used in the present study. B16F10 melanoma cells were pre-incubated with 0-5 μM MG132, 10-50 mM LiCl, 50 mM NaCl and KCl (negative control of LiCl) for 3 h, followed by co-incubation with TQ for 24 h.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT) assay. MTT assay was performed to confirm cell viability. B16F10 cells were dispensed to a 96-well plate ($0.5x10^5$ cells/well) and cultured at 37˚C for 24 h and were subsequently treated with 0-20 μM TQ (Sigma-Aldrich). Subsequently, 10 μl/well of MTT reagent I (10 mg/ml) were added followed by culture for 4 h at 37°C. Subsequently, solubilization solution MTT reagent II [10% sodium dodecyl sulfate (SDS) with 0,01 N HCl] was added followed by culture for 12 h at 37˚C. An ELISA reader (iMark microplate absorbance reader, #1681130; Bio-Rad) was used to measure the absorbance (550-600 nm) values.

Tyrosinase activity assay. Tyrosinase activity assay was performed to measure intracellular tyrosinase activity. The protease inhibitor, phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich) was mixed with pH 6.8 phosphate buffer containing 1% Triton X-100 to bring the concentration to 1 mM, and proteins were then extracted using lysis buffer. The extracted proteins were quantified by BSA assay and reacted with 0.5 mg/ml L-DOPA (Sigma-Aldrich) at 37˚C for 1 h. Tyrosinase activity was determined by measuring the ability of tyrosinase to degrade L-DOPA, a substrate, resulting in a purple substance. Subsequently, an ELISA reader (iMark microplate absorbance reader, #1681130, Bio-Rad) was used to measure the absorbance value at 450 nm. The image was scanned using a cannon MP276 scanner.

Measurement of the expression of β-catenin and tyrosinase. B16F10 cells were seeded in a 96-well plate and transfected with S37A or control vector using TurboFect™ transfection reagent. After 24 h, TQ (0-20 μM) was added to the cells for 24 h. The stimulated cells were washed with PBS and fixed with 3.5% paraformaldehyde for 30 min at 4˚C. FBS (1% in PBS) was used as a blocking reagent. The cells were incubated with anti-tyrosinase (sc-15341; Santa Cruz Biotechnology, Inc.) or anti-β-catenin (sc-7963; Santa Cruz Biotechnology, Inc.) polyclonal antibodies for 1 h at room temperature. Thecells were then subsequently washed with PBS and incubated with FITC-conjugated anti-mouse IgG (F0257; Sigma-Aldrich) or FITC-conjugated anti-rabbit IgG (F0382; Sigma-Aldrich) for 1 h at room temperature. The expression levels of tyrosinase or β-catenin were measured using a fluorometer (Flx800, BioTek Instruments, Inc.) at a 485 nm excitation wavelength and 535 nm emission wavelength.

Melanin content assay. Melanin content assay was performed to measure the intracellular melanin content. B16F10 cells ($0.5x10^5$) were solubilized in 1 ml of 1 N NaOH/10% DMSO for 2 h at 80˚C. The solubilized solution was centrifuged at 12,000 x g for 10 min at room temperature and the supernatants were transferred to fresh tubes. Subsequently, an ELISA reader (iMark microplate absorbance reader, #1681130, Bio-Rad) was used to measure the absorbance value at 470 nm.

Western blot analysis. Following the addition of protease inhibitor [10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 1 mM 4-(2-aminoethyl) benzensulfonyl fluoride] and phosphatase inhibitor (1 mM NaF, 1 mM Na_3VO_4) to buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, proteins were extracted using lysis buffer. These proteins were electrophoresed using a 9% SDS-polyacrylamide gel and transferred onto nitrocellulose (NC) film. After attaching the primary antibodies β-catenin (1:1,000; sc-7963; Santa Cruz Biotechnology, Inc.), MITF (1:1,000; ab140606; Abcam), tyrosinase (1:1,000; sc-15341; Santa Cruz Biotechnology, Inc.;), p-GSK3β (Cell Signaling Technology Inc.; #9339; 1:1000), GSK3β (1:1,000; sc-7291; Santa Cruz Biotechnology Inc.), proliferating cell nuclear antigen (PCNA; 1:1,000; sc-25280; Santa Cruz Biotechnology Inc.) and GAPDH (1:1,000; sc-166545; Santa Cruz Biotechnology Inc.) and the secondary antibodies (goat-anti-rabbit IgG, 1:2,000, ADI-SAB-300-J, Enzo Life Sciences; goat-anti-mouse IgG, 1:2,000, ADI-SAB-100-J, Enzo Life Sciences; rabbit-anti-goat IgG, 1:2,000, AP106P, EMD Millipore) for 2 h at room temperature. ECL (Daeillab Service Co., Korea) system was used for protein analysis. The quantification of protein expression was performed by densitometric analysis (ImageJ software, National Institutes of Health; NIH)

Reverse transcription-polymerase chain reaction (RT-PCR) and semi-quantitative PCR. Following treatment of the cultured B16F10 cells with the reagent, TRIzol reagent (Thermo Fisher Scientific, Inc.) was then used for RNA extraction. RT-PCR was performed on β-catenin, MITF, tyrosinase and GAPDH with RNA extracted using SuPrimeScript RT-PCR premix (GeNet Bio; #SR-8000). The primers used were as follows: β-catenin (50˚C, 25 cycle) sense 5'-GTGCAA TTCCTGAGCTGACA-3' and antisense, 5'-CTTAAAGAT GGCCAGCAAGC-3'; MITF (52˚C, 23 cycle) sense, 5'-GCT GGAAATGCTAGAATACA-3' and antisense, 5'-TGTTCA TACCTGGGCACTCA-3'; tyrosinase (55˚C, 23 cycle) sense, 5'-ATGGGTGTTGACCCATTGTT-3' and antisense, 5'-GCA CCATCTGGACCTCAGTT-3'; and GAPDH (55˚C, 23 cycle) sense, 5'-TGTTCCTACCCCCAATGTGT-3' and antisense, 5'-CCCTGTTGCTGTAGCCGTAT-3'. The DNA were separated on a 1% agarose gel and stained with ethidium bromide staining solution (#161-0433; Bio-Rad). The band was quantified using ImageJ software (NIH).

cDNA and siRNA transfection. TurboFect™ transfection reagent (Thermo Fisher Scientific, Inc.) was used to transfect the cells with scrambled siRNA, pCMV6, S37A cDNA, β-catenin siRNA and MITF siRNA. The siRNA (Shanghai Solarbio Bioscience & Technology Co., Ltd.) sequences used in this study were as follows: Scrambled siRNA, 5'-UUCUCC GAACGUGUCACGUTT-3'; β-catenin siRNA, 5'-GGGUUC

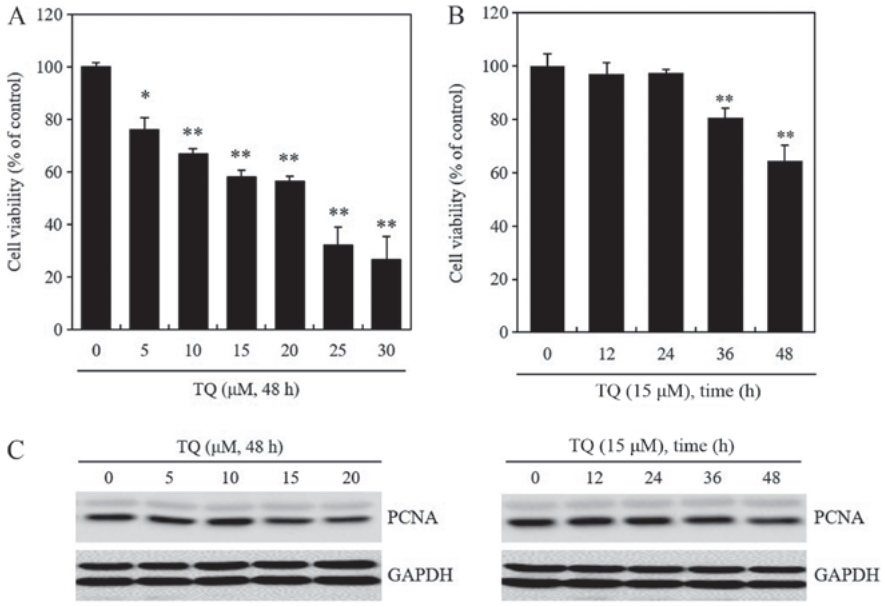

Figure 1. TQ treatment reduces the viability and proliferation of B16F10 mouse melanoma cells. Cell viability was determined by MTT assay. (A and C, left panel) B16F10 cells were treated with 5, 10, 15 and 20 μM of TQ for 48 h. (B and C, right panel) B16F10 cells were treated with 15 μM of TQ for 12, 24, 36 and 48 h. (C) PCNA expression was detected by western blot analysis. The expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. Values shown are the means ± SD; *P<0.05 and **P<0.01 compared to the untreated cells (n=3). TQ, thymoquinone; PCNA, proliferating cell nuclear antigen.

AGAUGAUAUAAAUd(TT)-3'; and MITF siRNA, 5'-AGC AGUACCUUUCUACCACd(TT)-3'. The transfected cells were then incubated for 6 h at 4˚C and subsequently treated with TQ (20 μM) or the vehicle (DMSO) for 48 h.

Immunofluorescence assay. Immunofluorescence assay was performed to confirm the differences in the intracellular expression of β-catenin and tyrosinase proteins in B16F10 melanoma cells. Following fixation with 3.5% paraformaldehyde for 20 min at room temperature, 0.1% Triton X-100 was used for 15 min at room temperature to increase the cell membrane permeability. Subsequently, β-catenin (1:100, sc-7963; Santa Cruz Biotechnology, Inc.) and tyrosinase (1:100, sc-15341; Santa Cruz Biotechnology, Inc.) primary antibodies were reacted for 2 h each at room temperature, while secondary antibodies with tetramethylrhodamine (TRITC; 1:100, T5393; Sigma-Aldrich) and fluorescein isothiocyanate (FITC; 1:100, F0382; Sigma-Aldrich) attached were reacted for 2 h at room temperature. The nuclei were stained using 4'6'-diamidi-no-2-phenylindole dihydrochloride (DAPI) for 20 min at room temperature. Subsequently, a BX51 fluorescence microscope (Olympus) was used to observe the cells.

Reagent treatment and heart-beating rate measurement of zebrafish. Zebrafish (3 to 12 months old) were used to obtain embryonic eggs. Males and females were separated in the chamber at a 2:1 ratio and kept in the dark for 12 h. Subsequently, most of the water in the chamber was removed and the fish were mated for 1 h. Using zebrafish embryo medium containing 55 mM NaCl, 0.174 mM KCl, 0.333 mM $CaCl_2{\cdot}2H_20$, 0.332 mM $MgS0_4$, and sodium bicarbonate in a 12-well plate, the zebrafish embryo eggs were treated with the reagent using TQ (0, 1, 2.5 and 5 μM) or PTU (200 μM, Sigma-Aldrich) at 10 h following fertilization and the medium was exchanged every 24 h. Dorsal and lateral images of zebrafish were acquired, and the concentration and distribution of melanin was quantified using an image J software (NIH). The zebrafish were anesthetized and euthanised by tricaine methane sulfonate (200-300 mg/l) by prolonged immersion (34-36). The euthanasia of zebrafish was confirmed by cardiac arrest. At 72 h following treatment with the reagents, a stereo microscope (cat. no. SMZ745T; Nikon) was used to measure the heartbeats of zebrafish juveniles for 3 min. The results were used to derive the mean beats per min.

Statistical analysis. The present study used the mean values from multiple rounds of experiments. One-way ANOVA statistical analyses at 95% confidence (with a post hoc Tukey's test) was performed. At a confidence interval (CI) of 5%, a P-value <0.05 was considered to indicate a statistically significant difference.

Results

TQ treatment reduces the viability and proliferation of B16F10 mouse melanoma cells. In the experiments in this study, the negative control represents the control that corresponds to the vehicle (DMSO) or mock (empty vector, scrambled siRNA). An MTT assay was performed to confirm whether treatment of the B16F10 mouse melanoma cells with TQ affected their viability. Following treatment with 5, 10, 15 and 20 μM of TQ for 48 h, a dose-dependent decrease in cell viability was observed (Fig. 1A). Additionally, following treatment with 15 μM of TQ for 12, 24, 36 and 48 h, a time-dependent decrease in cell viability was observed (Fig. 1B). The expression of PCNA, a marker of cell proliferation, was decreased following treatment with TQ at 15 and 20 μM for 48 h (left panel) and 15 μM for 24-48 h (right panel) (Fig. 1C). GAPDH

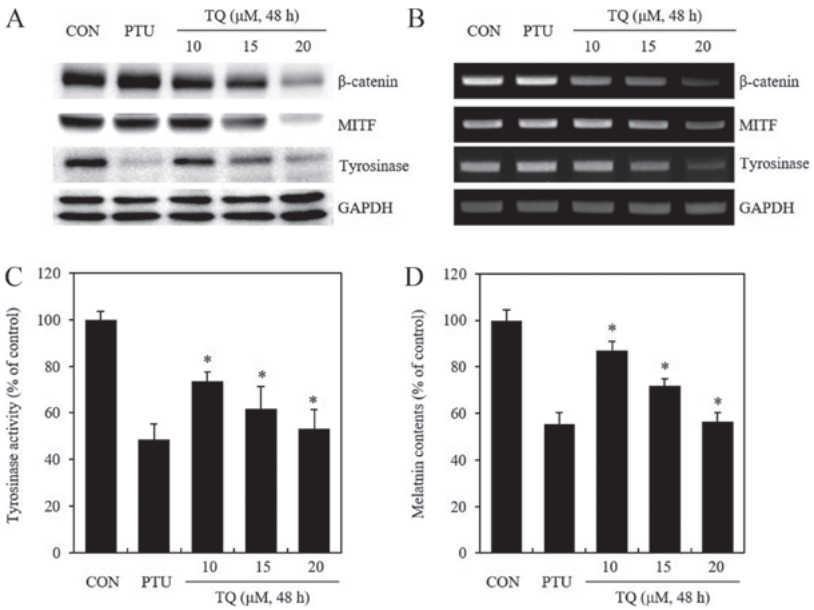

Figure 2. TQ treatment significantly inhibits protein expression and the transcription of β-catenin, MITF and tyrosinase in B16F10 mouse melanoma cells. B16F10 cells were treated with the indicated concentrations of TQ for 48 h. PTU (200 μM) was used as a positive control. (A) Expression of β-catenin, MITF and tyrosinase was determined by western blot analysis. (B) Expression of β-catenin, MITF and tyrosinase mRNA was detected by semi-quantitative RT-PCR. (C) Tyrosinase activity was determined by a tyrosinase activity assay. (D) Melanin contents were determined by a melanin content assay. The intracellular melanin content was confirmed by the color of the cell pellet (upper panel). Values shown are the means ± SD; *P<0.05 compared to control (n=5). TQ, thymoquinone; PTU, phenylthiourea; MITF, microphthalmias-associated transcription factor.

protein expression is represented by 2 sets of bands. In our laboratory and in other previous studies, GAPDH bands are represented by 2 sets of bands in western blot analysis (37-39). Therefore, this phenomenon, in which several bands of antibody are present, can be considered as the general reaction of the antibodies used. Unfortunately, the equipment with which to perform mass spectrometry was not available and thus this was not done (Fig. 1C). Taken together, these results confirmed that TQ treatment reduced the viability and proliferation of B16F10 mouse melanoma cells. At the concentration of 25 and 30 μM, TQ decreased cell viability decreased to more than the IC50 value (Fig. 1A), indicating a marked decrease in viability; in addition, at these concentrations, the majority of the had died. Thus, TQ was used at concentrations between 10-20 μM in the subsequent experiments. Although these concentrations were low, they effectively reduced the levels of the melanogenesis-related proteins MITF, tyrosinase and β-catenin (Fig. 2).

TQ treatment reduces the protein expression and mRNA transcription of β-catenin, MITF and tyrosinase, and this treatment also inhibits tyrosinase activity in B16F10 mouse melanoma cells. This study then examined whether TQ exerts an effect on MITF and tyrosinase, which are proteins associated with melanogenesis, as well as whether these proteins are regulated through the β-catenin pathway. PTU, a known inhibitor of melanogenesis, was used as a positive control. Following treatment with TQ for 48 h in a dose-dependent manner, western blot analysis was performed to confirm the expression of β-catenin, MITF and tyrosinase proteins (Fig. 2A). PTU, which is known to promote the intracellular degradation of tyrosinase proteins, only reduced the expression of tyrosinase protein. Conversely, TQ treatment resulted in a dose-dependent decrease in the expression of not only tyrosinase, but also of β-catenin and MITF proteins. Following treatment with PTU and TQ under the same conditions described above, differences in the levels of β-catenin, MITF and tyrosinase mRNA transcription were confirmed by semi-quantitative RT-PCR (Fig. 2B). PTU did not affect the level of β-catenin, MITF and tyrosinase mRNA transcription, while TQ treatment resulted in a dose-dependent decrease in the transcription of these genes. When tyrosinase activity was analyzed following treatment with PTU and TQ under the same conditions described above, TQ treatment led to a dose-dependent inhibition of tyrosinase activity (Fig. 2C). Herein, the decrease in the intracellular melanin content was examined by analyzing pigment deposits in cell pellets and the melanin content (Fig. 2D). On the whole, the results suggested that TQ inhibited both cell proliferation and melanogenesis. As shown in Fig. 1, the inhibitory effect of TQ on cell proliferation seemed to inhibit melanin production. Furthermore, as shown in Fig. 2D, the amount of melanin was quantified by the same number of cells and the amount of melanin was measured. This result confirmed that TQ decreased the synthesis of melanin in a concentration-dependent manner. In other words, TQ inhibited cell proliferation and also inhibited the synthesis of melanin. Following treatment with 15 μM of TQ in a time-dependent manner, the protein levels of β-catenin and MITF increased at 10 min to 6 h and decreased after 6 h. The mRNA levels were increased at 10 min to 1 h and decreased after 3 h. On the other hand, the mRNA levels of tyrosinase increased at 30 min and decreased after 3 h, whereas they decreased immediately after 10 min at the protein level, and increased at 30 min to 3 h. It was found that the mRNA and protein levels of β-catenin and tyrosinase were increased at up to approximately 6 h by TQ and were then downregulated

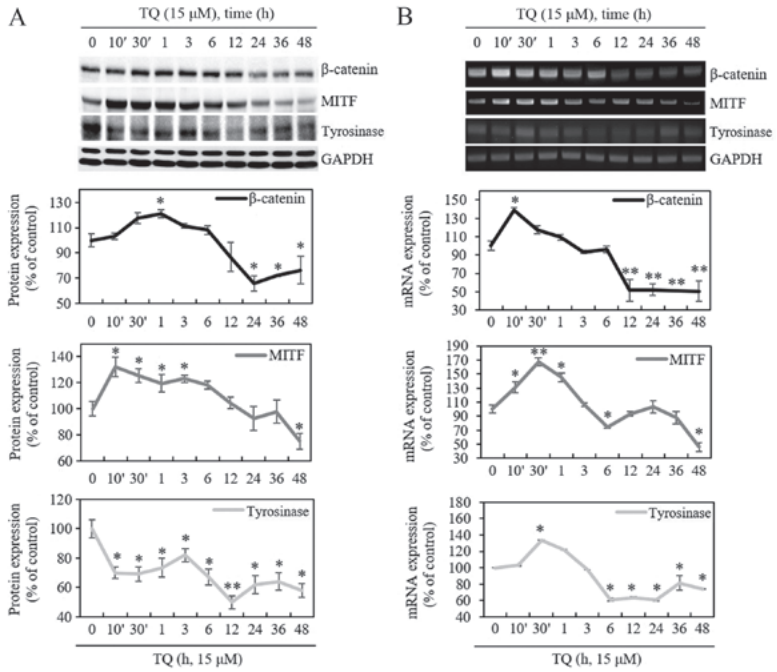

Figure 3. TQ regulates the protein expression and mRNA transcription of β-catenin, MITF and tyrosinase in B16F10 mouse melanoma cells. B16F10 cells were treated with 15 μM TQ for the specified time periods. (A) Expression of β-catenin, MITF and tyrosinase was determined by western blot analysis. (B) Expression of β-catenin, MITF and tyrosinase mRNA was detected by semi-quantitative RT-PCR. A treatment time of 0 h was used as a control, and protein expression or mRNA expression levels were quantified using ImageJ software (lower panels). Values shown are the means ± SD; *P<0.05 and **P<0.01 compared to untreated cells (n=4). TQ, thymoquinone; MITF, microphthalmias-associated transcription factor.

after 6 h. The expression of MITF protein was increased at up to approximately 3 h by TQ and was then downregulated after 6 h. However, the mRNA expression of MITF was increased at up to approximately 6 h by TQ and was then downregulated after 6 h. (Fig. 3A and B). In the subsequent experiments, the cells were treated with TQ for 48 h.

Pre-treatment with MG132 and LiCl results in the recovery of β-catenin and tyrosinase protein expression and activity initially reduced by TQ. To examine whether melanogenesis inhibited by TQ is regulated by β-catenin, 0,5, 1, 2.5 and 5 μM of MG132, a proteasome inhibitor, was used for treatment (Fig. 4A) and for pre-treatment at 3 h prior to TQ treatment. Subsequently, the expression of β-catenin, MITF and tyrosinase proteins (Fig. 4B) and tyrosinase activity were measured by tyrosinase activity assay (Fig. 4C). The expression of β-catenin, MITF and tyrosinase proteins, which was initially reduced by TQ, was recovered by pre-treatment with MG132, and tyrosinase activity also increased. The cells were also pre-treated with LiCl, a GSK3β inhibitor, at 10, 25, and 50 mM concentrations at 3 h prior to TQ treatment, and the expression of β-catenin, MITF, and tyrosinase proteins and tyrosinase activity were then measured (Fig. 4D and E). LiCl pre-treatment led to an increase in Ser-9 phosphorylated GSK3β protein, and as a result, the expression of β-catenin, MITF and tyrosinase proteins and tyrosinase activity, which were initially reduced by TQ, increased (Fig. 4D and E).

Overexpression of β-catenin increases MITF and tyrosinase protein expression, and activity initially reduced by TQ treatment. The expression of β-catenin, MITF and tyrosinase proteins was confirmed following treatment of the cells with TQ and transfection with 0.1, 0.2 and 0.3 μg of cDNA β-catenin overexpression vector with an Ala mutation at the β-catenin Ser-37 site (S37A). The results indicated that intracellular accumulation occurred due to the blockage of the ubiquitin-mediated proteasome degradation of β-catenin in B16F10 cells, while the expression of MITF and tyrosinase proteins increased (Fig. 5A). pCMV6 is an empty vector that was used as a negative control. To confirm whether the inhibition of melanogenesis by TQ is regulated by the β-catenin signaling pathway, the cells were treated with TQ following simultaneous transfection with S37A β-catenin and β-catenin siRNA. Subsequently, the expression of β-catenin, MITF and tyrosinase proteins (Fig. 5B) and the levels of tyrosinase activity were measured by tyrosinase activity assay (Fig. 5C). The expression of MITF that was reduced by TQ was increased by S37A mutant β-catenin and thus tyrosinase expression increased. This suggested that the accumulation of S37A mutant β-catenin, which is limited by proteasomal degradation, induced an increase in MITF and tyrosinase expression (Fig. 5B). The expression of β-catenin, MITF and tyrosinase proteins, and the levels of tyrosinase activity, that had increased subsequent to TQ treatment following transfection with S37A β-catenin, were reduced again by simultaneous transfection with β-catenin siRNA. Additionally, simultaneous transfection with MITF siRNA and S37A cDNA also reduced tyrosinase activity. Scrambled siRNA was used as a negative control (Fig. 5C).

An immunofluorescence assay was then performed subsequent to TQ treatment at 6 h following transfection with 0.3 μg of S37A β-catenin cDNA (Fig. 6A). The endogenous β-catenin and mutant form (S37A) of β-catenin in one cell are

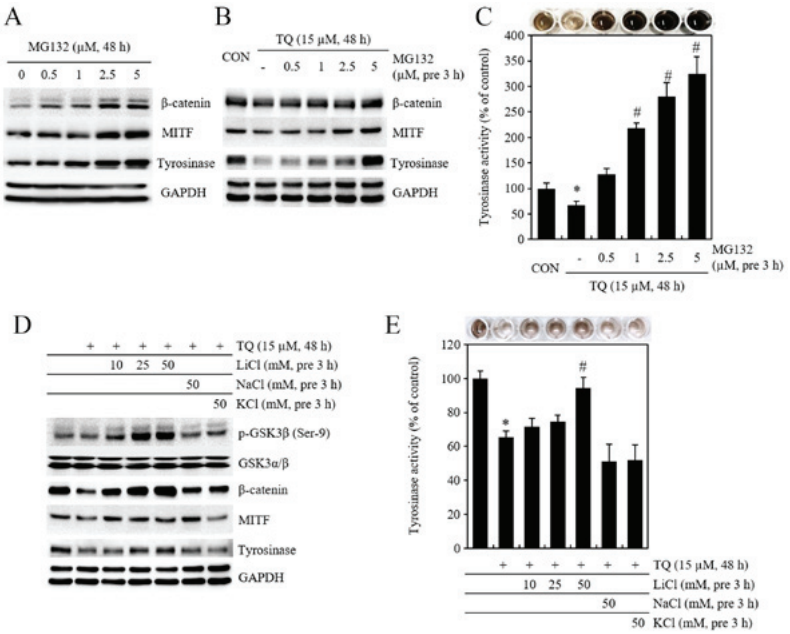

Figure 4. MG132 or LiCl treatment increases the protein expression of β-catenin, MITF and tyrosinase initially reduced by TQ. (A) B16F10 cells were treated with 0.5, 1, 2.5 and 5 μM of MG132. (B-E) B16F10 cells were treated with 15 μM TQ for 48 h in the absence or presence of MG132 or LiCl. NaCl and KCl were used as negative controls. (A, B and D) Expression of β-catenin, MITF, tyrosinase, GSK3α/β and levels of p-GSK3β (Ser-9) were detected by western blot analysis. (C and E) Tyrosinase activity was determined by a tyrosinase activity assay. Values shown are the means ± SD; *P<0.05 compared to the control; $^{\#}$P<0.05 compared to TQ-treated cells (n=3). TQ, thymoquinone; MITF, microphthalmias-associated transcription factor.

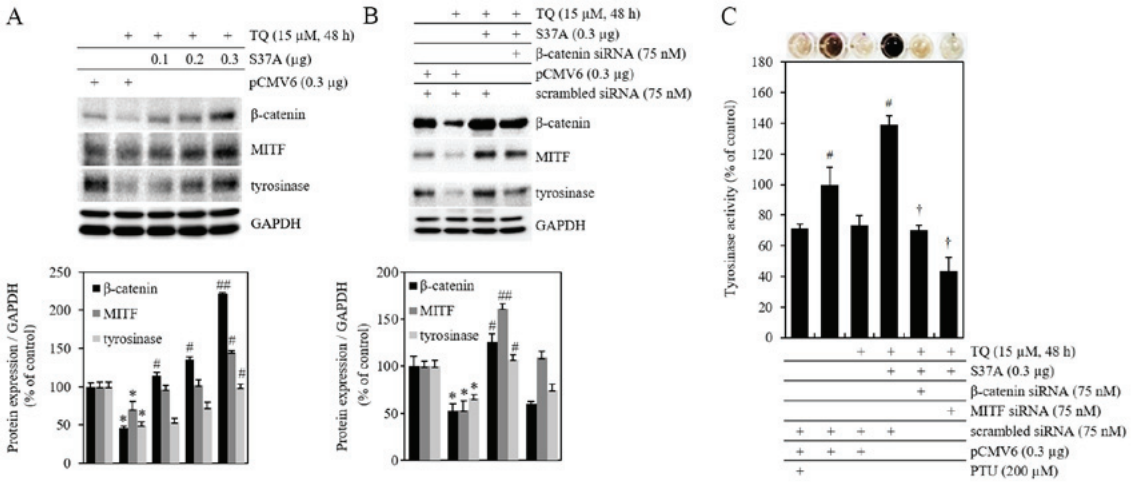

Figure 5. The mutant S37A β-catenin increases the protein expression of β-catenin, MITF and tyrosinase initially reduced by TQ. (A) B16F10 cells were transfected with S37A β-catenin cDNA in the absence or presence of 15 μM TQ for 48 h, and western blot analysis was performed. pCMV6 vector was used as negative control. (B) S37A cDNA and β-catenin siRNA were simultaneously transfected, and the expression of β-catenin, MITF and tyrosinase was detected by western blot analysis. Scrambled siRNA was used as a negative control. (C) S37A cDNA and β-catenin siRNA or MITF siRNA were simultaneously transfected, and tyrosinase activity assays were performed. Values shown are the means ± SD; *P<0.05 compared to the control (pCMV6 with or without scrambled siRNA-transfected cells); $^{\#}$P<0.05 and $^{\#\#}$P<0.01 compared to TQ-treated cells (n=7); †P<0.05 compared to TQ-treated cells in the S37A-transfected cells (n=4). TQ, thymoquinone; PTU, phenylthiourea; MITF, microphthalmias-associated transcription factor.

indistinguishable. However, cells with exogenous β-catenin are brighter than β-catenin in the surrounding cells. S37A used in this study was cDNA without a tag (Fig. 6A). The expression of tyrosinase (red) and β-catenin (green), that had been reduced by TQ, increased when the cells were transfected with S37A cDNA. A fluorometer was also used to quantify the expression of β-catenin and tyrosinase (Fig. 6B). The results confirmed that the inhibition of melanogenesis in B16F10 mouse melanoma cells by TQ was due to the inhibition of the β-catenin signaling pathway.

TQ inhibits melanogenesis in zebrafish. To determine the toxic effects of TQ on zebrafish, vital signs (movement, heartbeat and circulation) were measured as previously described (40). Zebrafish were also euthanized after anesthesia to determine the amount of melanin synthesis and euthanasia was confirmed by measuring vital signs (Fig. 7). At 72 h after the TQ or PTU treatment of zebrafish embryo eggs that had been fertilized for 10 h, heartbeats per min were measured (Fig. 7A). Additionally, dorsal and lateral images of zebrafish were acquired, and the concentration and distribution of melanin was quantified

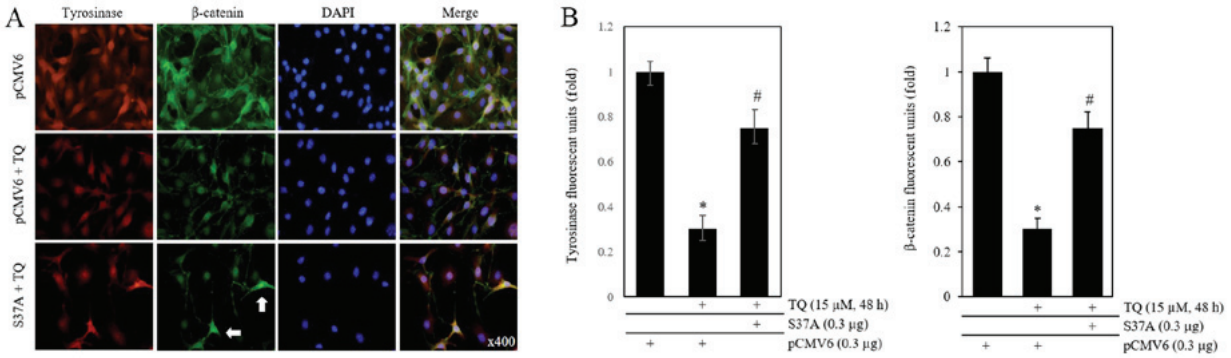

Figure 6. Transfection with S37A β-catenin increases the protein expression of tyrosinase initially reduced by TQ. (A) Tyrosinase (TRITC), β-catenin (FITC) and DAPI were simultaneously stained and analyzed by immunofluorescence microscopy. White arrows indicate cells transfected with the S37A β-catenin cDNA expression vector. (B) A fluorometer was used to quantify the expression of β-catenin and tyrosinase. Values shown are the means ± SD; *P<0.05 compared to the control (pCMV6-transfected cells); $^{\#}$P<0.05 compared to TQ-treated cells in the pCMV6-transfected cells.

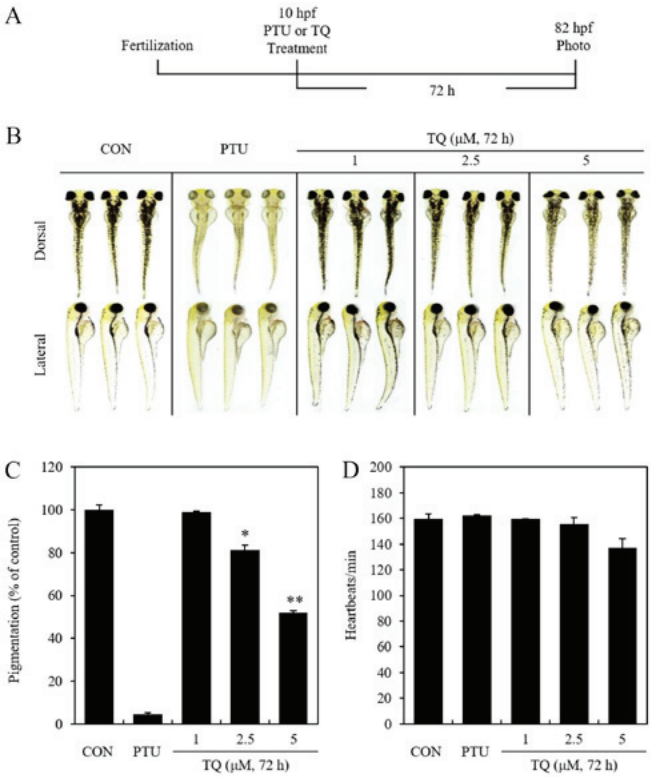

Figure 7. TQ treatment suppresses the melanogenesis of zebrafish. (A) Schematic diagram briefly illustrates the zebrafish experiments. At 10 h post-fertilization, zebrafish were treated with 1, 2.5 and 5 μM of TQ or 200 μM PTU for 72 h. PTU was used as a positive control. (B) At 72 h after the reagent treatment, zebrafish were photographed dorsally and laterally using a stereomicroscope. (C) The melanin content of the dorsal region was quantified using ImageJ software. (D) The zebrafish heartbeat rate was measured to confirm the toxicity of the reagent. Values shown are the means ± SD; *P<0.05 and **P<0.01 compared to the control (n=3). TQ, thymoquinone; PTU, phenylthiourea.

using imageJ software. (Fig. 7B and C). The results confirmed the dose-dependent inhibition of melanogenesis in zebrafish induced by TQ treatment. When heartbeats per min were measured in specimens treated with 200 μM PTU and 1, 2.5, or 5 μM of TQ to investigate the toxicity of TQ, the toxicity of TQ was confirmed at 5 μM, as indicated by a slight decrease in the heartbeats of the specimens treated with 5 μM of TQ (Fig. 7D). Given these observations, these results confirm that TQ treatment inhibits melanogenesis in zebrafish. Fig. 8 provides a schematic diagram detailing the control of melanin production through the β-catenin pathway regulated by TQ.

Discussion

Melanoma, which is the most aggressive and fatal form of skin cancer, occurs in melanocytes, the pigment cells found mainly in the skin. Incidences of malignant melanoma continue to increase at a steady pace. This malignancy can be treated with surgical excision at an early stage; however, once the disease advances to metastatic stages, treatment becomes extremely difficult. Recent discoveries involving the cell signaling and molecular mechanisms underlying this disease have provided a basic and deep understanding of melanoma

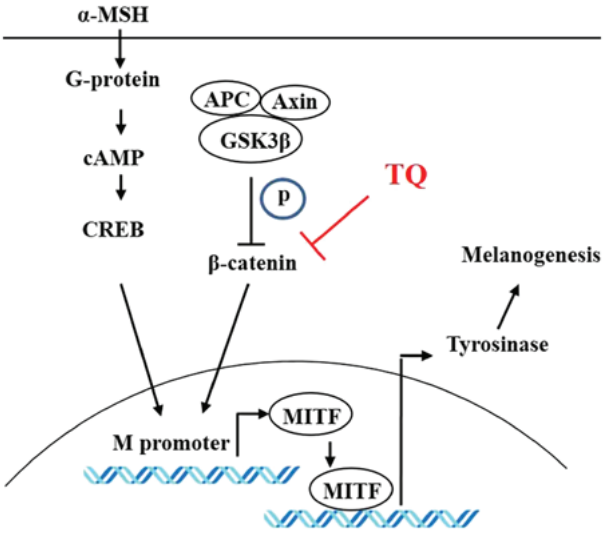

Figure 8. Schematic diagram illustrating the control of melanogenesis through the β-catenin pathway regulated by TQ. cAMP and Wnt/β-catenin pathways regulate the transcription of MITF. Inhibition of β-catenin by TQ inhibits the transcription of MITF, ultimately inhibiting the expression of tyrosinase, the melanogenesis protein. TQ, thymoquinone; MITF, microphthalmias-associated transcription factor; GSK3, glycogen synthase kinase 3; α-MSH, α-melanocyte stimulating hormone; CREB, cAMP response element binding protein; APC, adenomatous polyposis coli.

that is being used to develop targeted drugs and novel therapeutic methods (41).

TQ is an active ingredient isolated from the seeds of *Nigella sativa*, and since it was first extracted in 1960, its anticancer, anti-inflammatory and antioxidant activities have been investigated using both *in vitro* and *in vivo* models. The anti-inflammatory and antioxidant effects of TQ have been reported in various disease models, including encephalomyelitis, diabetes, asthma and cancer. The anticancer effects of TQ are mediated through various actions, including anti-proliferative actions, the induction of apoptosis, the termination of the cell cycle, the production of ROS, anti-metastatic actions and anti-angiogenic actions (42). The induction of ROS by TQ in breast cancer cells induces anti-proliferative effects (43). In human non-small cell lung cancer cells, TQ has been shown to inhibit proliferation and invasion through the ERK pathway (44). Hatiboglu *et al* (45) suggested that the inhibition of proliferation by TQ in B16F10 cells occurs via the inhibition of p-STAT3. Previously, in a study published by the authors, it was reported that TQ induces the apoptosis of rabbit chondrocytes (46). In this study, it was also confirmed that the proliferation of B16F10 cells was reduced by the inhibition of PCNA by TQ, and this study also identified the inhibition of melanogenesis and the mechanism of action of TQ in B16F10 mouse melanoma cells. In another previous study, researchers focused on effects, such as anticancer or anti-inflammatory effects mediated by TQ (31-33,43-45). However, this study focused on the identification of signal pathways regulated by TQ in melanogenesis in B16F10 cells. It was found that the β-catenin signaling pathway plays an important role in melanogenesis in B16F10 cells. These results are a novel finding which, to the best of our knowledge, has not yet been previously demonstrated.

The findings of this study suggested that TQ inhibited both cell proliferation and melanogenesis (Figs. 1 and 2). Therefore, when the cells were stained with DAPI, the number of cells was lower in the cells treated with TQ than in the control cells, since all of the experiments revealed a quantitative result in proportion to the number of cells (Fig. 6A). In this study, experiments were conducted using B16f10 melanocytes, but not with normal cells. It has previously been demonstrated that thymoquinone inhibits cell proliferation and induces apoptosis even in normal cells (46). That is, TQ may exert an inhibitory effect on melanogenesis and may suppress the proliferation of cancer cells; however, it may also have a side-effect of inhibiting the proliferation of normal cells. The inhibition of melanogenesis by TQ treatment was confirmed through a decrease in the intracellular melanin content and tyrosinase transcription, protein expression and activity. To examine whether the inhibition of melanogenesis by TQ in B16F10 mouse melanoma cells involved the β-catenin pathway, 3 independent experiments were conducted.

First, pre-treatment with MG132 was applied to block the degradation of β-catenin by the ubiquitin-mediated proteasome pathway. The expression of β-catenin, that was previously decreased by TQ treatment, increased inside the cells due to degradation being blocked by MG132, and this also resulted in an increased MITF and tyrosinase expression and tyrosinase activity. Proteasomal degradation occurs inside the cells via the phosphorylation of the Ser-33, 37 and 45 and Thr-41 sites of β-catenin by GSK3β, which is an upstream pathway (16,17). The phosphorylation of the Tyr-216 site of GSK3β increases the enzymatic activity of GSK3, while the phosphorylation of the Ser-9 site of GSK3β induces inactivation (47). Lithium (Li^+) is known to cause the inactivation of this pathway by inducing the phosphorylation of the Ser-9 site of GSK3β (48). Accordingly, pre-treatment with LiCl was applied in this study, and this resulted in an increased phospho-GSK3β expression that led to the blockage of the proteasomal degradation pathway of β-catenin, and increased the expression and activity of tyrosinase. Finally, the cells were transfected with S37A β-catenin cDNA to induce the overexpression of β-catenin. The amino acids 32-37 of β-catenin (Asp-Ser-Gly-Ile-His-Ser) comprise a motif that is recognized by the β-TrCP E3 ubiquitin ligase, and

the phosphorylation of Ser-33 and 37 is essential for recognition by the E3 ligase (18). S37A β-catenin exhibits a blocked phosphorylation of the Ala-33 site of GSK3β and the β-TrCP E3 ubiquitin ligase cannot recognize the ligase motif, ultimately resulting in intracellular accumulation due to the degradation pathway being blocked by the proteasome. Consequently, the recovery of MITF and tyrosinase expression and tyrosinase activity that were initially reduced by TQ was confirmed. When the cells were simultaneously transfected with S37A β-catenin and β-catenin siRNA, β-catenin expression again decreased, while the expression of MITF and tyrosinase, and the activity of tyrosinase also decreased (Fig. 5). These results indicated that β-catenin is an important protein for the expression and activity of tyrosinase.

The MITF promoter that is located most proximal to the common downstream exons is known as the M promoter and appears to be selectively expressed in melanocytes. The MITF-M promoter is targeted by several transcription factors that are important in neural-crest development and signaling (19). At the MITF-M promoter, Wnt/β-catenin signaling is crucial for the differentiation of melanocytes from the neural crest. In embryogenesis, the Wnt/β-catenin signaling pathway plays a deterministic role in the formation of body parts in the early embryo stage and is also involved in gastrulation and blastopore lip formation (49). The overexpression of β-catenin or the inhibition of translation by GSK3 antisense mRNA injection in zebrafish can lead to the formation of an extra blastopore and body axis (50). When the same concentration of TQ used to treat the B16F10 cells was used to treat zebrafish embryo eggs, this treatment caused deformations or death during embryogenesis (data not shown). Accordingly, zebrafish eggs were treated with TQ concentrations that were reduced 3-15-fold from the cell treatment concentration, and as a result, a reduction in melanogenesis was confirmed (Fig. 7). Additional studies, however, are warranted to determine whether the inhibition of melanogenesis in zebrafish by TQ is mediated via the β-catenin pathway.

In conclusion, the findings of this study demonstrated that TQ inhibited melanogenesis in B16F10 mouse melanoma cells by inhibiting the β-catenin signaling pathway, and it also impeded melanogenesis in zebrafish. The findings of the present study may prove to be useful for the future development of therapeutic agents targeting pigmented lesions and melanoma.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

HJ and SMY conceived and designed the study and the experiments, performed research, and wrote the manuscript. SJK conceived and designed the study and the experiments, conducted research, and wrote the manuscript.

Ethics approval and consent to participate

This research conformed to the guidelines of and was approved by the Kongju National University Ethical Review Board.

Patient consent for publication

Not applicable.

Competing interests

The authors confirm that they have no competing interests.

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