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Inhibitory Effect of *Prunus persica* Flesh Extract (PPFE) on Melanogenesis through the Microphthalmia-associated Transcription Factor (MITF)-mediated Pathway

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Abstract – Novel tyrosinase inhibitors are important for pigmentation in the skin. Following extraction of tyrosinase inhibitors from edible vegetables or fruits, we found that the *Prunus persica* flesh extract (PPFE) exhibited potential inhibitory activity for melanogenesis. PPFE showed tyrosinase inhibitory activity in an enzymatic assay and PPFE also significantly inhibited the melanin formation in cultured mouse melan-a cells. Moreover, real-time RT-PCR analysis revealed that the inhibition of melanin production by PPFE was closely related to marked suppression of mRNA expression of tyrosinase and tyrosinase-related protein-1 and -2 (TRP-1 and TRP-2) in melan-a cells. Further investigation found that the modulation of tyrosinase expression by PPFE was associated with the transcriptional regulation of the microphthalmia-associated transcription factor (MITF). PPFE inhibited the promoter activity of MITF and suppressed MITF mRNA expression in melan-a cells. These results indicate that PPFE down-regulates melanogenesis-associated gene expression through MITF-mediated transcriptional regulation and these events might be related to the hypopigmentary effects of PPFE.
Keywords – *Prunus persica*, melanin, tyrosinase, MITF, melan-a cell

Introduction

Melanin synthesis, or melanogenesis, occurs in melanocytes through an enzymatic process that is catalyzed by at least three enzymes: tyrosinase, tyrosinase-related protein-2 (TRP-2), and tyrosinase-related protein-1 (TRP-1) (Hearing *et al*., 1991). Tyrosinase (EC 1.14.18.1) is the key enzyme associated with the undesirable browning of fruits and vegetables, in addition to the coloring of skin, hair and eyes in animals (Kubo *et al*., 2000; Perez-Gilabert *et al*., 2001). Tyrosinase catalyzes the oxidation of L-tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) and further leads to DOPA quinone formation, which is the initial step in melanin synthesis (Shin *et al*., 1998). Since melanin has many functions, this enzymatic oxidation of L-tyrosine to melanin is an important step. Indeed, alterations of melanin synthesis occur in many disease states. Therefore, tyrosinase inhibitors have become increasingly significant in the food industry as well as in medicinal and cosmetic products (Mosher *et al*., 1983; Parvez *et al*., 2006). A number of tyrosinase inhibitors have been reported from natural or synthetic sources, but only a few are being used as depigmenting agents. The major limitation is primarily due to various safety concerns. For example, linoleic acid, hinokitiol, kojic acid, naturally occurring hydroquinones, and catechols were reported to inhibit the enzyme activity, but these compounds also exhibited side effects (Seo *et al*., 2003). In addition, several phytochemicals have been reported as tyrosinase inhibitors including α-arbutin (Maeda *et al*., 1996; Curto *et al*., 1999; Virador *et al*., 1999), ellagic acid (Yoshimura *et al*., 2005), oxyresveratrol (Kim *et al*., 2002), chlorophorin and norartocarpanone (Shimizu *et al*., 1998).

Microphthalmia-associated transcription factor (MITF) plays an important role as a transcriptional activator of genes encoding melanogenesis-related proteins including tyrosinase and tyrosinase-related protein (TRP) (Eves *et al*., 2006; Costin *et al*., 2007). MITF is a basic helix-loop-helix leucine zipper dimeric transcription factor belonging to the MYC superfamily of proteins (Hallsson *et al*., 2004). MITF forms dimers and binds to specific sequence motifs present in the promoter of its target genes to activate their transcription. Various studies have documented

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the role of MITF in the induction of genes required for melanin formation as well as normal melanocyte development. It regulates the transcription of three major pigmentation enzymes which are tyrosinase, TRP-1, and TRP-2 (also known as dopachrome-tautomerase, DCT (Bently *et al*., 1994; Hemesath *et al*., 1994; Yasumoto *et al*., 1994).

In our program to develop inhibitors of melanin synthesis from natural products including plant extracts and edible vegetables or fruits, we found the *Prunus persica* flesh extract (PPFE) exhibited the inhibition of tyrosinase activity as well as melanin formation in a melan-a cell culture system. Although the biological activities of the seeds (Fukuda *et al*., 2003) and flowers (Kim *et al*., 2002) of *Prunus persica* have been reported extensively, the inhibitory activity of PPFE on melanogenesis in addition to the study on its mechanism of action have not been fully investigated. Further, we investigated whether the inhibition of melanin formation by PPFE is associated with the transcriptional regulation of key enzymes by using an MITF reporter assay and gene expression in cultured melan-a cells.

We report herein for the first time the inhibitory effects of PPFE on melanogenesis, including the decreased expression of MITF, tyrosinase, TRP-1, and TRP-2.

Experimental

Plant materials and reagents – The fruits of *P. persica* L. Batsch (peaches) were purchased from a peach supplier in Janghowon, Gyeonggi-Do, Korea. The samples were authenticated by Dr. N.S. Seong at the National Institute of Crop Science, RDA, Korea. RPMI medium was supplied from Cambrex Co. (Wakersville, MD). Fetal bovine serum (FBS), trypsin-EDTA solution (1X), and antibiotic-antimycotic solutions were from GIBCO-BRL (Grand Island, NY). iQTM SYBR® Green Supermix was purchased from Bio-Rad Co. (Hercules, CA). Arbutin were TPA, Mushroom tyrosinase, L-DOPA, and α-arbutinwere purchased from Sigma Chemical Co. (St. Louis, MO).

Extraction – The fruits were washed with tap water, and its pericarp and seed were removed. The collected flesh (100 g) was then extracted three times with five volumes (w/v) of 80% ethanol for total extract of 48 h at room temperature. The resulting extracts were filtered and then concentrated to dryness at 40 °C under vacuum to produce the EtOH extract (30.4 g). The resulting EtOH extract was suspended in water, and was partitioned with ethyl acetate (EtOAc), n-butanol (BuOH), and H_2O, successively to give EtOAc (6 g), BuOH (2 g) and H_2O-soluble fractions (22.4 g), respectively. The yield of dried extract exhibited 30.4%, 2.0%, 6.0%, and 22.4% of the original sample with the EtOH extract, BuOH, EtOAc, and H_2O-soluble fractions, respectively. The dried extracts were stored at –20 °C until needed.

Enzyme assay – L-DOPA was used as a substrate and tyrosinase (EC 1.14.18.1) activity was monitored by dopachrome formation at 475 nm for the appropriate time (usually not longer than 10 min). The assay was performed according to the procedure of Masatomo et al (1980) with minor modifications. Briefly, the preincubation mixture consisted of 1.8 ml of 0.1 M phosphate buffer (pH 6.8), 0.6 ml of water, 0.1 ml of the sample solution, and 0.1 ml of the aqueous solution with mushroom tyrosinase (130 units). After preincubation for 5 min at room temperature, L-DOPA solution (0.4 ml of 6.3 mM) was added and then the reaction was monitored at 475 nm for 3 min.

Cell line and culture – Melan-a cells (originally established by Dr. Bennett at the University of London) were kindly provided by Skin Research Institute, Amore-Pacific Co., Korea. Melan-a cells were grown in RPMI 1640 medium supplemented with antibiotics, 10% fetal bovine serum (FBS), and 20 nM TPA. The cells were incubated at 37 °C in a humidified atmosphere of 10% CO_2. Each experiment was carried out at least three times.

Inhibitory effect of melanin biosynthesis using cultured mouse melan-a cells -Confluent cultures of melan-a cells were rinsed with Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS) and lysed with 0.25% trypsin/EDTA. Cells were plated into 6-well plastic culture plates at a density of 3×10^4 cells/well. At 72 h after plating, the media were replaced with or without (control) test sample. After an additional 72 h incubation, the adherent cells exposed to the test samples were assayed.

Determination of melanin content in melan-a cells – The melanin content was determined as follows: after removing the media and washing the cells with Ca^{2+} and Mg^{2+}-free PBS, the cell pellet was dissolved in 0.3 ml of 1N NaOH, and incubated at 60 °C for 10 min. The optical density at 475 nm was measured by an ELISA reader (Bio-Rad, Hercules, CA). The melanin content was calculated using a standard curve with melanin.

Real-time RT-PCR – Real-time RT–PCR was employed to determine the gene expression of tyrosinase, tyrosinase-related proteins (TRPs) and MITF in melan-a cells. Briefly, total RNA was extracted using the TRI reagent and reverse transcribed at 42 °C for 60 min in 20 μl of reverse transcription system (Promega, Madison, WI) with 0.5 μg of oligo $(dT)_{15}$ primer. The levels of mRNA

for tyrosinase, tyrosinase-related proteins (TRPs) expression were conducted on a MiniOpticon system (Bio-Rad, Hercules, CA), using 5 μl of reverse transcription product, iQTM SYBR® Green Supermix (Bio-Rad, Hercules, CA) and primers in a total volume of 20 μl. The standard thermal cycler conditions were employed: 95 °C for 20 s before the first cycle, 95 °C for 20 s, 56 °C for 20 s, and 72 °C for 30 s, repeated 40 times followed by 95 °C for 1 min and 55 °C for 1 min.

Specific tyrosinase primers were designed using the Roche Applied System (Basel, Swiss) and provided by Bioneer Corporation (Seoul, Korea). The following sequences were used: tyrosinase (accession No. NM011661) forward, 5'-CACCCTGAAAATCCTAACTTACTCA-3'; tyrosinase reverse, 5'-CTCTTCTGATCTGCTACAAATG ATCT -3'; TRP-1 (accession No. NM031202) forward, 5'-TGGGAACACTTTGTAACAGCA-3'; TRP-1 reverse, 5'-ACTGCTGGTCTCCCTACATTTC-3'; TRP-2 (accession No. X63349) forward, 5'-GGCTACAATTACGCCGTTG-3'; TRP-2 reverse, 5'-CACTGAGAGAGTTGTGGACCAA-3'; β-actin (housekeeping gene; accession No. NM007393) forward, 5'-AAGGCCAACCGTGAAAAGAT-3'; β-actin reverse, 5'-GTGGTACGACCAGAGGCATAC-3'. Real-time PCR was conducted on a MiniOpticon system (Bio-Rad, Hercules, CA) using 5 μl of reverse transcription product, iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA) and primers in a total volume of 20 μl. The standard thermal cycler conditions were employed: 95 °C for 20 s before the first cycle, 95 °C for 20 s, 56 °C for 20 s, and 72 °C for 30 s, repeated 40 times followed by 95 °C for 1 min and 55 °C for 1 min.

For quantitative PCR, amplifications of the MITF gene were performed using iQ™ Supermix (Bio-Rad, Hercules, CA), gene-specific TaqMan PCR probes and primers, and a standard thermal cycler protocol (95 °C for 5 min before the first cycle, 95 °C for 10 sec and 58 °C for 30 sec, repeated 39 times). For specific MITF probes and primers of PCR amplifications, the following primers were designed using Metabion (Martinsried, Germany): MITF sense, 5'-TGATCCCCAAGTCAAATG-3'; MITF antisense, 5'-TTC TTCTGTCGGTTTTCA-3'; MITF probe, FAM-CTCGTT GCTGTTCCCGTTGC-BHQ1, β-actin (housekeeping gene) sense, 5'-ACAGCTTCTTTGCAGCTCCTTC-3'; β-actin antisense, 5'-CGACCAGCGCAGCGATATC-3', β-actin probe, HEX-CACACCCGCCACCAGTTCGCCAT-BHQ1.

The threshold cycle (CT), indicating the fractional cycle number at which the amount of amplified target gene reaches a fixed threshold from each well, was determined by using MJ Opticon Monitor software. Relative quantification, representing the change in gene expression from real-time quantitative PCR experiments between the PPFE group and untreated control group was calculated by the comparative CT method as published earlier (Livak *et al*., 1996). The data were analyzed using the equation $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = [CT \text{ of target gene} - CT \text{ of housekeeping gene}]_{\text{treated group}} - [CT \text{ of target gene} - CT \text{ of housekeeping gene}]_{\text{untreated control group}}$. For the treated samples, evaluation of $2^{-\Delta\Delta CT}$ represents the fold change in gene expression, normalized to a housekeeping gene (β-actin) and relative to the untreated control.

Transient transfection and dual luciferase assays – Melan-a cells were plated in 24 wells and incubated at 37 °C. pMITF-Gluc reporter system harboring the promoter region (494 bp) of MITF was kindly provided by Amore Pacific R&D Center (Seoul, Korea). pGL3-FL was obtained from S. Oh (Inje University, Busan, Korea). At 40 - 50% confluency, the cells were washed with PBS and the gaussian luciferase reporter construct pMITF-Gluc (0.6 μg) and the control *Firefly* luciferase vector (pGL3-FL) (30 ng) were transfected for 24 h using Transpass D2 reagent (New England BioLabs, Ipswich, MA). Our preliminary study showed that the transfection reaction for 24 h did not affect the cell viability of melan-a cells. The transfected cells were treated with PPFE. After 24 h incubation, cell lysates were prepared and determination of gaussia and firefly luciferase activities were performed using the gaussia luciferase assay kit (New England BioLabs, Ipswich, MA) and luciferase reporter assay system (Promega, Madison, WI) respectively, according to the manufacturers' protocols and were measured using a luminometer (MicroLumat *Plus*, Berthold Technologies, Dortmund, Germany). Relative luciferase activity was calculated as the ratio of gaussia luciferase activity to *firefly* luciferase activity. Data are representative of at least three independent experiments.

Statistical analysis – Statistical analysis was performed using SigmaStat 2.03 (SPSS, Chicago, IL). One-way analysis of variance (ANOVA) was used to compare mean responses among the treatments. Statistical probability of $P < 0.05$ was considered significant.

Results and Discussion

In the development of depigmenting agents, modulation of the biosynthetic pathway for melanin can be considered as a primary target. One of the well-known targets in this pathway is tyrosinase. Tyrosinase is a rate-limiting enzyme in melanin formation and catalyzes the first step of oxidation of L-DOPA. On the line, many depigmenting agents were screened by inhibition of this

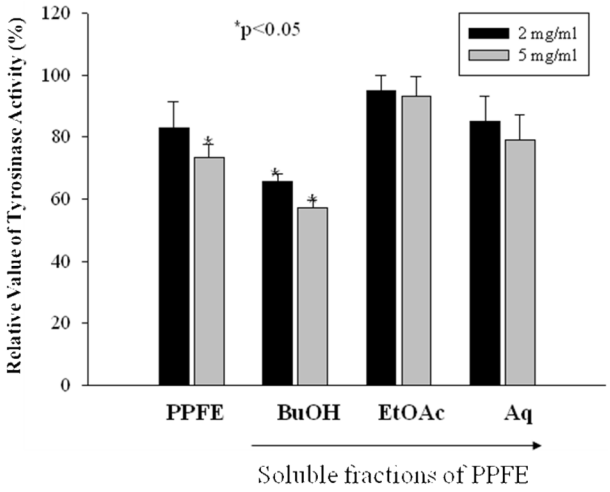

Fig. 1. Tyrosinase inhibitory activity of PPFE, as well as the BuOH, EtOAc and aq-soluble fractions of *P. persica* flesh. Statistical analyses were performed using a Student's *t* test and one-way ANOVA. Data are expressed as the mean ± SD of four determinations. $*P < 0.05$ was considered statistically significant.

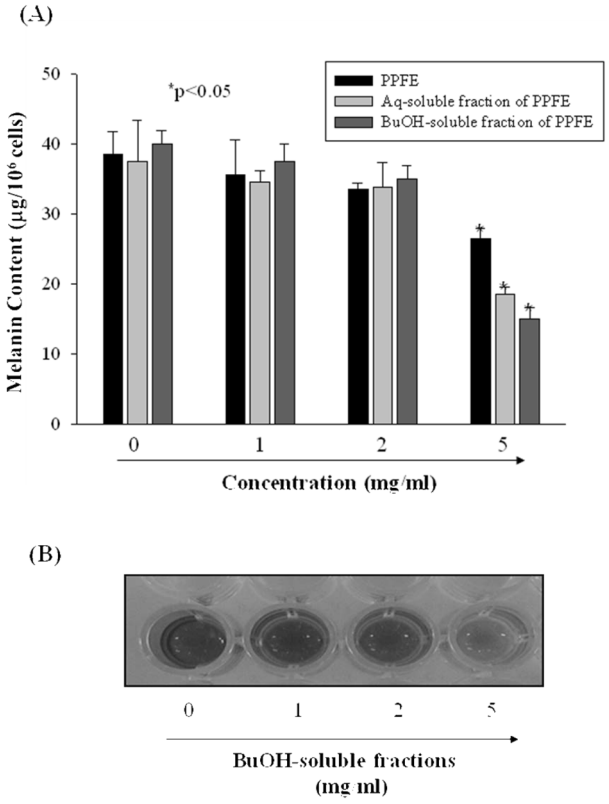

Fig. 2. (A) Effects of PPFE, BuOH and Aq-soluble fractions of *P. persica* flesh on melanin content. (B) Macroscopic views of the BuOH-soluble fraction of PPFE on melanin content. Data shown are representative of three independent experiments. Statistical analyses were performed using a Student's *t* test and one-way ANOVA. Data are expressed as the mean ± SD of four determinations. $*P < 0.05$ was considered statistically significant.

enzyme's activity. In our program to develop depigmenting agents from natural products, including edible fruits or vegetables, we employed an inhibition assay for tyrosinase activity as a primary screening system. When evaluated with test samples derived from *P. persica* flesh, inhibitory activity to tyrosinase was found to be 26.5%, 43.1%, 7.0%, and 21.0% in the PPFE (EtOH-soluble total extract), BuOH, EtOAc and Aq-soluble fractions, respectively, at the test concentration of 5 mg/ml as shown in Fig. 1. The BuOH-soluble fraction of PPFE showed the most potent inhibitory activity among the test extracts. The inhibitory activity by the BuOH-soluble fraction was dose-dependent as demonstrated by 12.5%, 34.5%, and 43.1% reductions in activity at test concentrations of 1, 2, and 5 mg/ml, respectively. These results indicate that PPFE has potential tyrosinase inhibitory activity which might be useful in anti-browning and depigmenting agents (Briganti *et al*., 2003). To our knowledge, this is the first report showing the inhibitory activity of tyrosinase by PPFE.

Further effects on melanogenesis by PPFE were then evaluated with cell-based assay systems. We used melan-a cells for determination of melanin content. As shown in Fig. 2 (A), the inhibition of melanogenesis by PPFE-, Aq-, and BuOH-soluble fractions was 31.0%, 51.5%, and 62.4%, respectively, at a test concentration of 5 mg/ml. Similar to the findings with tyrosinase activity, the BuOH-soluble fraction exhibited was most potent, and the inhibitory activity towards melanin formation was dose-dependent (Fig. 2 (B)). To determine the effects of *P. persica* flesh on the gene expression of tyrosinase, melan-a cells were treated with PPFE and the BuOH-soluble fractions for 24 h and mRNA levels for tyrosinase were analyzed by real-time RT-PCR. In agreement with the data shown in Fig. 2, treatment with the BuOH-soluble fractions resulted in a significant and dose-dependent down-regulation of gene expression for tyrosinase (Fig. 3(A)). These results suggest that *P. persica* flesh can inhibit melanin synthesis in mouse melan-a cells through the down-regulation of tyrosinase mRNA.

Prunus persica has been used for traditional medicines (Kim *et al*., 2002; Ministry of Health and Welfare, Japan, 2001). The seeds are frequently used as an ingredient in a variety of Chinese medicine prescriptions for the treatment of women's diseases. The major chemical constituents of the herb include the cyanogenic glycosides, amygdalin and prunasin (Ministry of Health and Welfare, Japan, 2001). Multiflorin B is an active component of the flowers of *P. persica* and has been used for skin disorders in East Asia, even in ancient times (Kim *et al*., 2002).

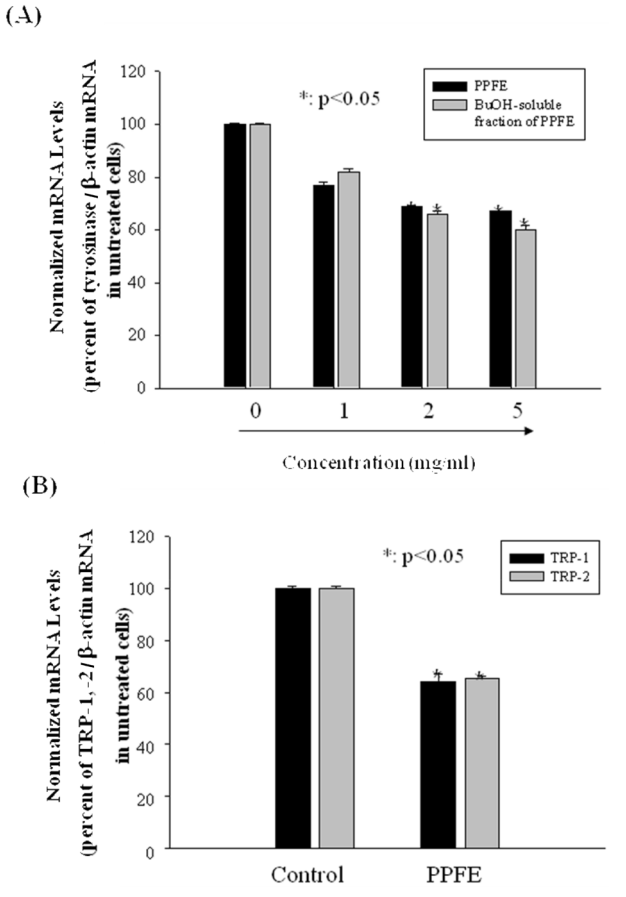

Fig. 3. PPFE and the BuOH-soluble fraction inhibits mRNA expression of tyrosinase (A), TRP-1 and TRP-2 (B) in melan-a cells. Melan-a cells were treated with PPFE and the BuOH-soluble fraction for 24 h. The mRNA levels of tyrosinase, TRP-1 and TRP-2 were determined by real-time RT-PCR as described in Materials and Methods. Statistical analyses were performed using a Student's *t* test and one-way ANOVA. Each value represents the mean ± SD for three different experiments performed in triplicate. *$P < 0.05$ was considered statistically significant.

Other chemical constituents of *P. persica* are saccharides, organic acids, vitamins, polyphenols, pectins, and amino acids (Carbonaro *et al*., 2002). Our research group reported that PPFE attenuates chemotherapy-induced hepatotoxicity in mice (Lee *et al*., 2008), improves chemotherapeutic efficacy and protects against nephrotoxicity in cisplatin-treated mice (Lee *et al*., 2009). In addition, we also reported the pericarp extract of *P. persica* attenuates chemotherapy-induced acute nephrotoxicity and hepatotoxicity in mice (Lee *et al*., 2008). However, none of the extracts or chemical components of *P. persica* have yet been reported for the inhibition of melanin formation in melanocytes. The present data show that the extracts of *P. persica* flesh exhibit inhibition of melanin formation and mRNA expression of tyrosinase in a melan-a cell culture system.

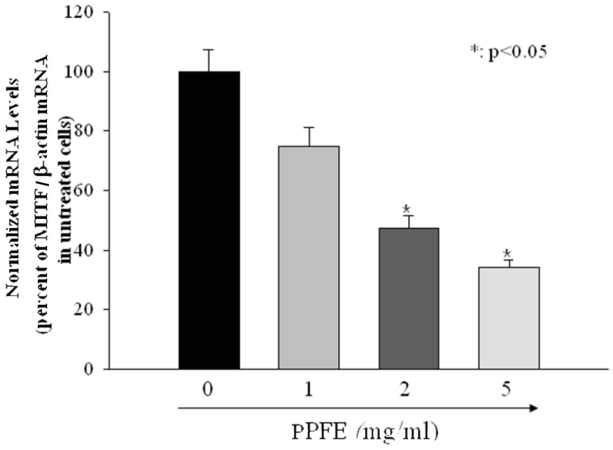

Fig. 4. PPFE down-regulates the mRNA expression of MITF in mouse melan-a cells. Melan-a cells were treated with the indicated concentrations of PPFE (1, 2 and 5 mg/ml) for 24 h. The mRNA levels of MITF were determined by quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Using the $2^{-\Delta\Delta CT}$ method as described in Materials and Methods, the data are expressed as the fold change in gene expression normalized to a housekeeping gene, β-actin and relative to the untreated control (*first lane*). Data shown are mean ± SEM of four determinations. *Statistically significant compared with the control cultures ($P < 0.05$).

In addition, recent studies suggest that tyrosinase-related proteins (TRP) (del Marmol *et al*., 1996) as well as tyrosinase are also considered important melanogenic enzymes and at least two isozymes, TRP-1 (DHICA oxidase) (Kuzumaki *et al*., 1993) and TRP-2 (DOPAchrome tautomerase) (Tsukamoto *et al*., 2000) have been reported. We also found that PPFE down-regulated the gene expression of TRP-1 and TRP-2 at the test concentration of 5 mg/ml as shown in Fig. 3 (B). Although the Persica extracts are able to inhibit melanin synthesis by the regulation of tyrosinase and TRPs, the mechanism of action is still unclear. One plausible transcription factor microphthalmia-associated transcription factor (MITF) is known to regulate the expression of tyrosinase and TRPs by binding to the M-box promoter of these proteins (Yasumoto *et al*., 1997; Aksan *et al*., 1998) and thus initiate the transcription of genes required for melanin synthesis (Widlund *et al*., 2003). Therefore, we determined whether the down-regulation of melanosomal proteins were regulated at the transcriptional level. The effects of PPFE on the expression of MITF mRNA in mouse melan-a cells were measured by quantitative real-time RT-PCR, also known as fluorogenic 5' nuclease assay technology using gene-specific TaqMan® probes and primers. As indicated in Fig. 4, treatment of melan-a cells with increasing concentrations of PPFE (1, 2 and 5 mg/ml) markedly reduced MITF expression, indicating that

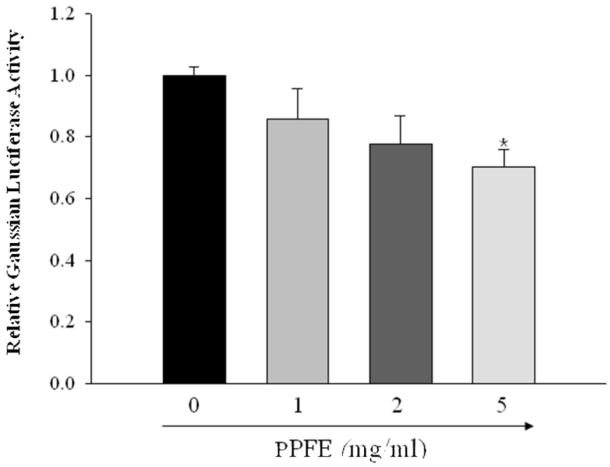

Fig. 5. PPFE decreases MITF transactivation in mouse melan-a cells as measured by transient transfection and dual luciferase assays. Melan-a cells were transfected with a gaussia luciferase reporter construct, pMITF-Gluc plasmid and treated with increasing concentrations of PPFE (1, 2 and 5 mg/ml) for 24 h. The cells were also co-transfected with the *Firefly* luciferase control vector (pGL3-FL) to normalize transfection rates. Values represent the mean ± SEM of four determinations. *Statistically significant as compared with control cultures ($P < 0.05$).

MITF transcription is inhibited by PPFE.

The transcription factor MITF plays a key role in pigmentation. To better understand the inhibitory molecular mechanism of melanin synthesis, we examined the effect of PPFE on MITF promoter activity. MITF-dependent transcription was measured in mouse melan-a cells transfected with a gaussia luciferase reporter plasmid containing the MITF promoter region (494 bp). PPFE significantly down-regulated transactivation of MITF as measured by a dual luciferase assay (Fig. 5). In accordance with our proposed mechanism of action of PPFE, several compounds, including 4,4'-dihydroxybiphenyl, hinokitiol and (−)-epigallocatechin-3-gallate were also recently reported to reduce melanin synthesis through the regulation of MITF production (No *et al*., 2006; Choi *et al*., 2006; Kim *et al*., 2004). Also, several kinases have been found to phosphorylate specific serine residues (i.e., 73, 298 and 409) of MITF. Moreover, the phosphorylation of MITF at S73 by ERK and at S409 by ribosomal S6 kinase (RSK)-1 (an ERK downstream kinase) has been associated with the ubiquitin-mediated degradation of MITF (Wu *et al*., 2000; Goding *et al*., 2000; Xu *et al*., 2000).

Further study is needed to determine whether the activation of ERK signaling is associated with the modulation of MITF by the extracts or active components of *P. persica* flesh.

In conclusion, this study suggests that PPFE could be used to alter skin pigmentation and thus be considered as promising candidates for treating or even preventing hyperpigmentation as well as using PPFE components as depigmenting agents. The present investigation revealed for the first time that *P. persica* flesh has the potential to inhibit melanin synthesis through the down-regulation of tyrosinase, TRP-1, and TRP-2 mRNA expression. The down-regulation of this key enzyme in melanogenesis by PPFE was also associated with the modulation of the transcription factor MITF.

Abbreviations: $_{L}$-DOPA, $_{L}$-3,4,-Dihydroxyphenylalanine; TPA, 12-O-Tetradecanoylphorbol-13-acetate; TRP, Tyrosinase related protein; MITF, Microphthalmia transcription factor; Real-time RT-PCR, real-time reverse transcriptase-polymerase chain reaction

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