의학박사 학위논문

Anti-graying effect of the extract of *Pueraria thunbergiana* via potentiating the cAMP/MITF-M pathway

cAMP/MITF-M 경로 활성화를 통한 갈근추출물의 백모방지효과 연구

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지도 교수 정 진 호

이 논문을 의학박사 학위논문으로 제출함

2014년 07월

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박원석의 의학박사 학위논문을 인준함

2014년 07월

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Abstract

Anti-graying effect of the extract of *Pueraria thunbergiana* via potentiating the cAMP/MITF-M pathway

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Introduction:

Gray hair is the most typical symptom of hair aging and follicular melanocyte plays important role in hair graying. MITF-M is a crucial transcription factor regulating the survival, proliferation, migration and melanin synthesis in follicular melanocyte.

This study aimed to find novel natural materials which can suppress hair graying via regulating cAMP/MITF-M signaling pathway.

Methods:

To find active materials for hair graying, we observed the melanin and cAMP synthesis in cultured melanocytes. We also examined the melanogenesis activity with newly constructed luciferase reporter system and western blot for monitoring cAMP/MITF-M signaling. Then, the melanin synthesis and anti-hair graying effect were elucidated with zebrafish and $MITF^{vit/vit}$ mice.

Results:

The extract of *Pueraria thunbergiana* (PT extract) and its active compound, puerarin were found to up-regulate MITF transcription via increasing intracellular cAMP level. Furthermore, the PT extract and puerarin stimulated the expression of tyrosinase, TRP-2 and Bcl-2 proteins resulting the melanin synthesis and survival of melanocytes. The treatment of PT extract and puerarin on zebrafish treated with by 1-phenyl-2-thiourea and chronologically gray-haired $MITF^{vit/vit}$ mice recovered the pigmentation in two hypo-pigmentation models.

Conclusion:

The PT extract and puerarin stimulate the melanogenesis via cAMP/ MITF-M signaling pathway *in vitro*, and they can prevent the follicular depigmentation and vitiligo by stimulating the melanin synthesis.

Keywords: hair graying, MITF, Melanogenesis, *Pueraria thunbergiana*

Student Number: 2005-31203

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INTRODUCTION

The hair follicle is a complex mini-organ involving interactions from epithelial (different keratinocyte lineages, endothelium), mesenchymal (dermal papilla cells, connective tissue sheath cells) and neuroectodermal cell populations (nerves, melanocytes) [1]. Melanocytes located in the basal layer of the epidermis are broadly similar to those located in the basal layer of the hair follicle infundibulum. A much neglected second follicular melanocyte population is located amongst the basal sebocytes of the sebaceous gland. A third sub-population is located in the mid portion of the hair follicle outer root sheath. The most proximal follicular melanocyte sub-population, and the only one to contribute to pigmentation of the hair shaft, is located in the hair bulb above and around the mid-upper follicular papilla. Melanocytes of the epidermis, hair follicle bulb (the only melanogenic region of the follicle) and hair follicle outer root sheath differ in many important ways (Figure 1) [2].

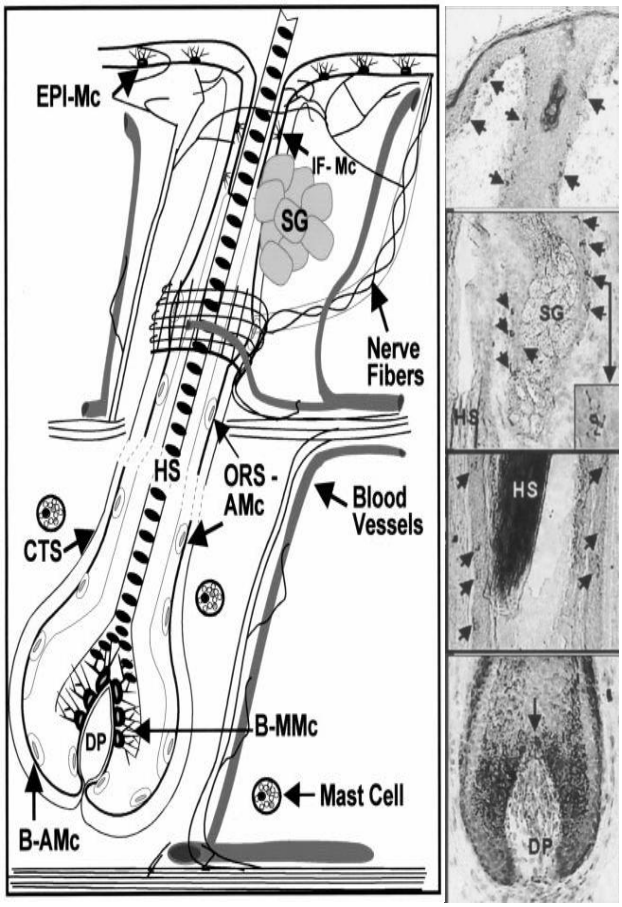


Figure. 1. Schematic and histological representations of the distribution of melanocytes in different regions of the human anagen scalp hair follicle.

Hair graying is one of the typical signs of human aging, and the maintenance of hair pigmentation is dependent on the presence and functionality of follicular melanocytes (Figure 2). Hair graying would be integrated results of interconnected mechanisms, which originated from the large interindividual variations in the predominant relevant key factors, such as 1) oxidative damage [3,4], 2) hair follicular melanocytes stem cell damage/ defective self-maintenance [5], 3) insufficient growth factors (MITF, SCF, bFGF, HGF etc), and 4) abnormal melanosome transfer [6].

In melanocytes and melanoma cells, melanogenesis is controlled by a cascade of enzymatic reactions regulated at the level of tyrosinase and its related proteins (TRP-1, TRP-2). This tyrosinase enzyme synthesizes dopaquinone from tyrosine and appears to control the rate-limiting step of melanogenesis. Melanin is synthesized in the melanosomes of mammalian melanocytes for photoprotection from ultraviolet radiation [7]. Melanin synthesis is stimulated by a large number of effectors, including 1-oleyl-2-acetyl-glycerol [8], ultraviolet B radiations [9], cAMP-elevating agents (forskolin, IBMX, α-MSH), and GR [10].

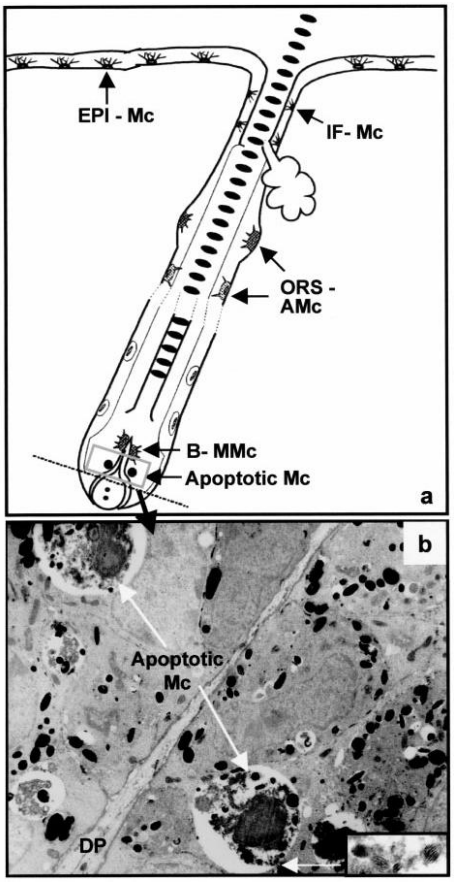
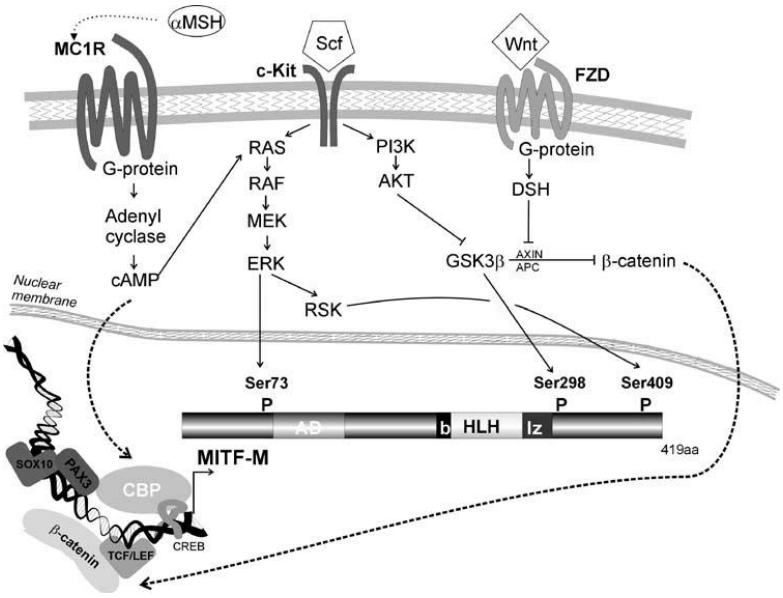


Figure. 2. Schematic representation of early catagen hair follicle showing loss of some bulbar melanotic melanocytes via apoptosis.

(A)



(B)

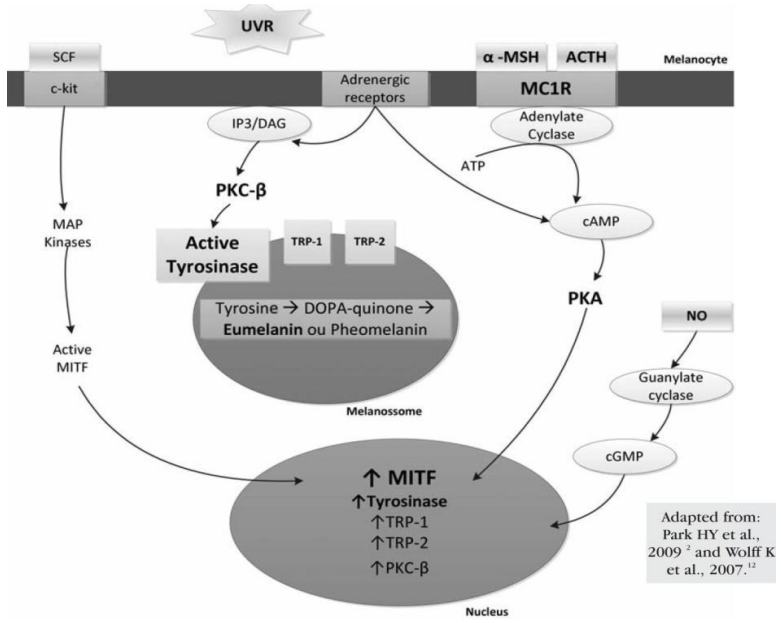


Figure. 3. (A)Transcriptional and post-translational regulation of the MITF gene and protein, and (B) Regulations of melanin synthesis.

cAMP pathway plays a key role in the regulation of melanogenesis by augmenting the enzyme activity of pre-existing tyrosinase and increasing tyrosinase mRNA synthesis (Figure 3A) [11]. cAMP, through activation of protein kinase A (PKA) and CRE binding protein (CREB) transcription factor, promotes the expression of microphthalamia-associated transcription factor (MITF) [12], a melanocyte-specific transcription factor crucial for melanocytes development and differentiation [13,14]. As a result, MITF binds to and activates the tyrosinase promoter, leading thereby to stimulation of melanogenesis (Figure 3B) [15-17].

A number of agents, including α-melanocyte stimulating hormone (α-MSH), forskolin, isobutylmethylxanthine, cholera toxin [12], phosphatidylinositol 3-kinase (PI3K) inhibitor (LY294002) [18], the lysosphingolipid, sphingosylphosphorylcholine (SPC) [19], and glycyrrhizin [20] upregulate Mitf expression with the subsequent induction of tyrosinase expression in melanoma and melanocytes.

The cAMP-elevating agents activate the protein kinase A (PKA) pathway to phosphorylate Serine 133 of cAMP response element-binding (CREB) protein. Activated CREB binds the cAMP response element (CRE) consensus motif located between 140 and 147 bp from the transcription initiation site of the Mitf-M promoter to upregulate the Mitf gene expression [12]. Independently of PKA, cAMP activates the MAP kinase cascade result mediates phosphorylation of Mitf at serine 73 and serine 409 by ERK and Rsk-1

respectively, and the subsequent post-translational downregulation of Mitf by degradation [21].

(A)

(B)

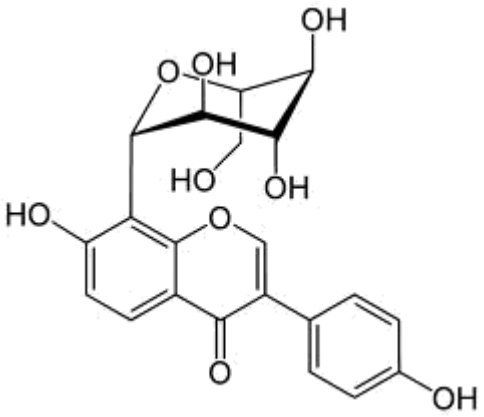


Figure. 4. Characteristics of (A) *Pueraria thunbergiana* (family *Leguminosae*) and (B) puerarin (7, 4′ -dihydroxyisoflavone-8 β -glucopyranoside).

Pueraria thunbergiana (family *Leguminosae*, Figure 4A) has been frequently used as an oriental traditional medicine or a food [22]. *P. thunbergiana*, of which main components are puerarin and daidzin [23, 24], is used for counteracting problems associated with alcohol liver injury [25], bone loss [26] and menopause. Many researchers reported that these pharmacological effects may originate from its isoflavones of *P. thunbergiana* [27, 28]. Puerarin (7, 4′-dihydroxyisoflavone-8β-glucopyranoside, Figure 4A) is a major active ingredient of *P. thunbergiana* Radix. It has been reported that puerarin has therapeutic effects on hypertension [29], cerebral ischemia [30], myocardial ischemia [31], diabetes mellitus [32] and arteriosclerosis [33]. The molecular mechanism involved puerarin's ability is believed to act as a scavenger of reactive oxygen species and antioxidants [34].

In this study, the extract of *P. thunbergiana* and its active compounds, puerarin were investigated to stimulate the melanogenesis via cAMP/ MITF-M signaling pathway in vitro and prevent the follicular depigmentation in hair cycle-accelerated hair graying mouse model.

MATERIALS AND METHODS

Materials

All chemicals were obtained from Sigma (St. Louis, MO). The ethanol paste extracts of *Pueraria thunbergiana* was kindly provided from Bioland Co. (Cheonan, Korea). $Mitf^{vit/vit}$ mice were obtained from the Jackson Laboratory (Bar Harbor, Maine).

Cell culture

Melan-a murine immortalized melanocytes were cultured in RPMI1640 with 10% fetal bovine serum and penicillin/ streptomycin (100IU/ 50 μg per mL) in a humidified atmosphere containing 10% CO_2 in air at 37 ℃.

Measurement of melanin content

The melanin content of cultured melan-a cells was measured in accordance with the method described by Oka et al. [35]. Briefly, the melan-a cell pellets were solubilized in 1N NaOH for 2 hr, then the supernatant was analyzed at a spectrum of 405nm. To examine the true melanin formation from the same number of cells, the total melanin content of each pellet was divided by the number of melanocytes.

cAMP immunoassay

Melanocytes were lysed in 0.1 M HCl to inhibit phosphodiesterase activity and centrifuged at 2,000 g for 15 min. The concentration of cAMP was

measured using the cAMP assay kit in accordance with the manufacture's instructions (Biomol International, Plymouth, PA) and expressed as pmol per ml [36].

Reporter assays

The human MITF-M(494bp, GenBank Seq. D82874), tyrosinase(390bp, GenBank Seq. M27160), TRP-2(683bp, GenBank Seq. L38953) promoters were cloned & amplified from human genomic DNA(Clontech Laboratories, CA) with polymerase chain reaction, and their inserts were ligated into the EcoR1/BamH1 site of pGluc-basic plasmid(New England Biolabs, Ipswich, MA). For reporter positive control, pCMV-Gluc (New England Biolabs, Ipswich, MA) was used.

The reporter constructs were transfected into melan-a cells using Fugene™ 6 according to the manufacturer's instruction (Roche Molecular Biochemicals, Indianapolis, IN). Forty-eight hours post-transfection, cells were treated with test materials for 12hr or otherwise as indicated. Secreted gaussia luciferase were assayed using BioLux™ Gaussia Luciferase Flex Assay Kit according to manufacturer's instructions (New England Biolabs, Ipswich, MA).

Western blot analysis

The cultured melanocytes treated with or without IBMX or test materials were homogenized in ice-cold homogenization buffer containing 50 mM Tris-base (pH 7.4), 150 mM NaCl, 10 mM EDTA, 0.1% Tween-20, and protease inhibitors (0.1 mM PMSF, 5 μg/ml aprotinin, and 5μg/ml leupeptin). Equal

amounts of extracted proteins (30 μg) were resolved using 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with anti-CREB, phospho-CREB (rabbit polyclonal; Cell Signaling Technology, Beverly, MA), MITF (mouse monoclonal; Abcam, Cambridge, UK), tyrosinase, Bcl-2 and TRP-2 (rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies, diluted 1:1000 in blocking solution, overnight at 4℃. The membranes were further incubated with anti-rabbit or anti-mouse HRP-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and treated with an enhanced chemiluminescence solution (Pierce, Rockford, IL). The signals were captured on an Image Reader (LAS-3000; Fujifilm, Tokyo, Japan) and analyzed with densitometry software (Image J®). For a loading control, the same membranes that had been stripped and stained with Coomassie Brilliant Blue.

Stimulation of melanogenesis of zebrafish treated with by 1-phenyl-2-thiourea

Synchronized embryos were collected and arrayed by pipette, three to four embryos per well, in 96-well plates containing 200 μℓ embryo medium. Test materials were dissolved in 0.1% DMSO, then added to the embryo medium from 35 to 60 hrs (25 hrs exposure). The effects on the pigmentation of zebrafish were observed under the stereomicroscope. Occasional stirring as well as replacement of the medium were done daily to ensure the even distribution of the test materials. In all experiments, 0.2 mM 1-phenyl-2-thiourea (PTU) was used to generate transparent zebrafish without interfering

the developmental process [37], and considered as a standard negative control. Phenotype-based evaluations of body pigmentation were carried out at 60 hr. For observation, embryos were dechorionated by forceps, anesthetized in tricaine methanesulfonate solution (Sigma, St. Louis, MO), mounted in 3% methyl cellulose on a depression slide (Aquatic Eco-Systems, Apopka, FL, USA), and photographed under the stereomicroscope MZ16 (Leica Microsystems, Ernst-Leitz-Strasse, Germany) [38].

Evaluation of suppression of hair graying effects on MITF$^{vit/vit}$ mice depilated repeatedly

The telogen-phase pelages of the MITF$^{vit/vit}$ mice were plucked with finger tips at 12 weeks after birth to force new hair cycle generation. This manipulation turned the coat color of Mitf$^{vit/vit}$ mice into gray, earlier than the normal 6 months after birth. After topical application of test sample for 3 weeks, global photographs were taken and the pelages of the mice were re-plucked to further develop the gray color [39]. The protease Esperase 8.0L (Sigma, St Louis, MO) is used to measure the melanin content in the hairs. A reaction buffer is prepared by dissolving Esperase in a buffer (50 mM Tris-HCl, 5 mM DTT, pH 9.3) to a concentration of 10 NPU/mL. 10 mg of mouse hair is added to 1 mL of the reaction buffer, and the reaction is carried out at 37℃ for 13 h, while shaking at 1,000 rpm. Thereafter, the hair is instantly separated from the reaction solution by centrifugation, and then the OD_{400} per mg of the hair reaction was measured for the determination of the hair melanin content.

Statistical evaluation

Averages ± SE of the means were calculated; statistical analysis of results was performed by Student's t test for independent samples. Values of *$p<0.05$, **$p<0.001$ and ***$p<0.0001$ were considered significant.

RESULTS

PT extract and puerarin induced melanogenesis in melanocytes

To observe whether PT extact and puerarin induces melanogenesis in melan-a cells, the melanin contents of cultured melanocytes were determined with treatment of 50μM puerarin and 25 and 50 μg/ml of PT extract for 72h. As shown in Figure 5A, puerarin and PT extract significantly induced the melanin contents in melan-a cell compared to the basal level in untreated cells ($P<0.01$), particularly PT extract increased the melanogenesis with dose-dependent manner.

PT extract and puerarin increased the cellular cAMP level

cAMP play a key role in regulation of skin and hair pigmentation by activating the MAP kinase cascade. Effects of puerarin and PT extract on cellular cAMP level were examined in cultured melanocytes treated with 50μM puerarin and 50 μg/ml of PT extract for 0.5 and 1h. Puerarin and PT extract significantly increased the cellular cAMP levels of melanocytes ($P<0.01$) compared to non-treated control (Figure 5B), and more than IBMX, a cAMP-elevating agent. The cellular cAMP level increased time dependently.

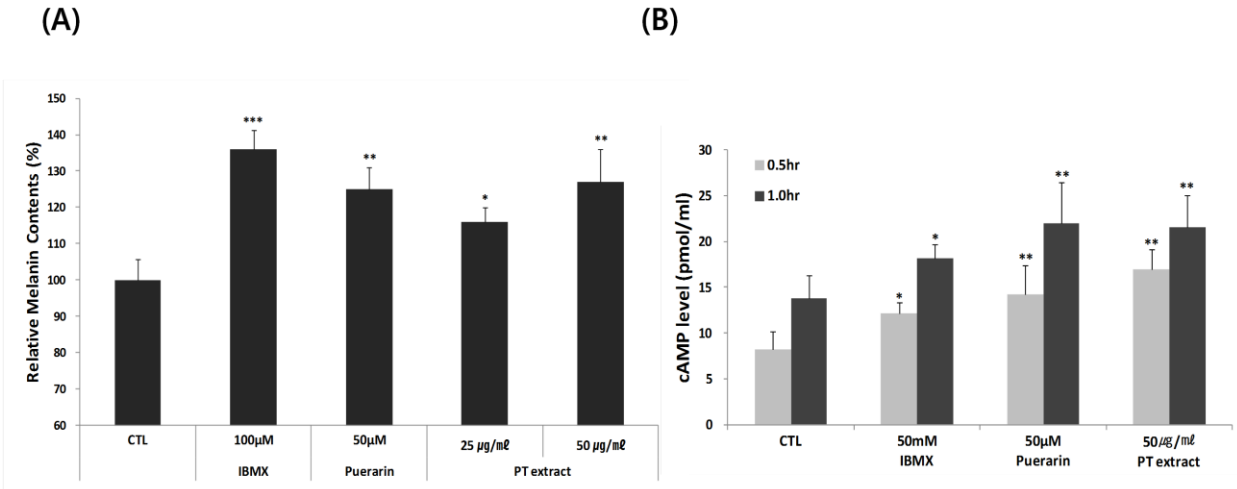


Figure 5. (A) Effect of puerarin and PT extract on melanin content in melan-a cells. After incubation of melan-a cells with 100μM IBMX, 50μM puerarin and two concentration (25 to 50 μg/ml) of PT extract were applied for 72 h, after which the relative contents of melanin (control value taken as 100% in each case) were presented as mean ± SE. (B) The effect of puerarin and PT extract on cellular cAMP of melan-a cell. After incubation of melan-a cells with 50mM IBMX, 50μM puerarin and 50 μg/ml of PT extract for 0.5 and 1 h, cAMP contents are presented as mean ± SE. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

PT extract and puerarin stimulated the transcriptional activity of pMITF-Gluc

The activities of the constructed promoter reporter assays of MITF, Tyrosinase and TRP-2 in melan-a cell were confirmed with 12h treatment of α-MSH, IBMX and db-cAMP. As shown in Figure 6A, the constructed reporter system of MITF, tyrosinase and TRP-2 were detected stable in melan-a cell lines transfected with plasmids containing each gene promoter at upstream of the Gaussia luciferase coding sequence in pGLUC-basic. To examine whether puerarin and PT extract induce the transcriptional activity of melanogenesis related genes, 50μM of puerarin and 25, 50μg/ml of PT extract were treated to the reporter assay systems for 12 hour, 100μM IBMX as a positive control. As shown in Figure 6B, 50uM puerarin ($P<0.01$) and 50 μg/ml PT extract ($P<0.05$) induced significant increase in MITF promoter activity respectively, however, did not work directly in tyrosinase and TRP-2 promoter (data not shown).

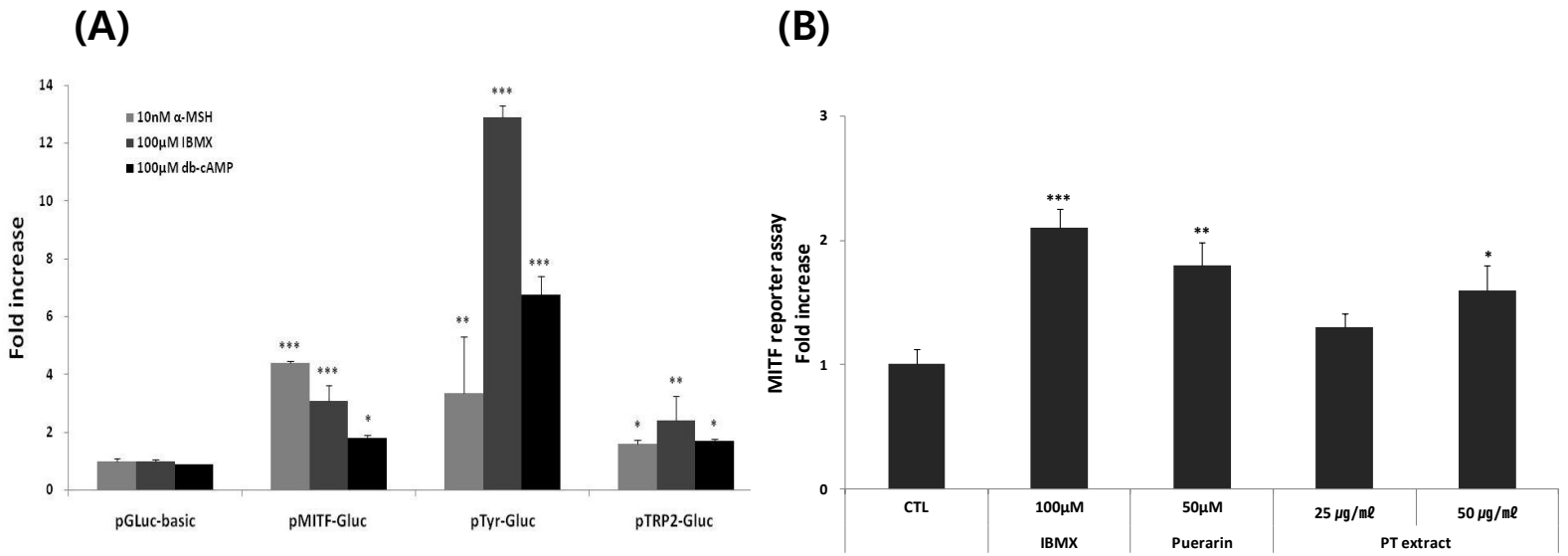
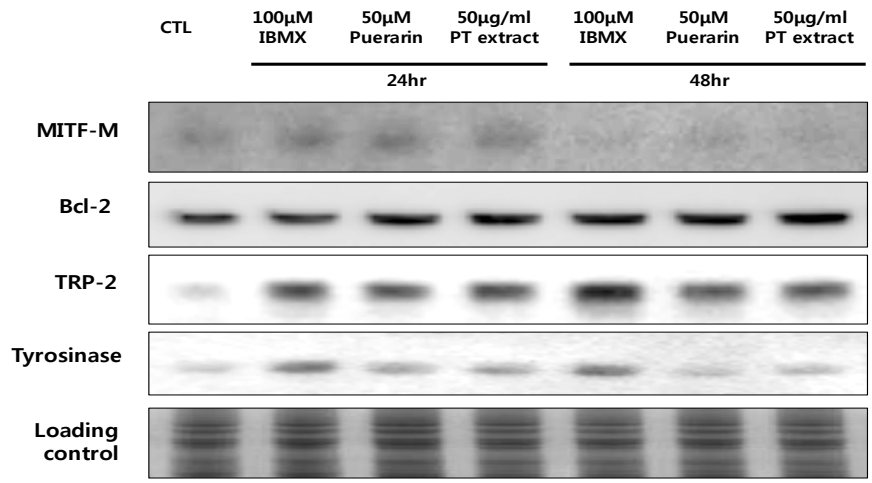


Figure 6. (A) Construction of promoter reporter assays of MITF, tyrosinase, TRP-2 in melan-a cell. Mitf, tyrosinase, TRP-2 promoter activity was detected in stable melan-a cell lines transfected with plasmids (containing each gene promoter upstream of the gaussia luciferase coding sequence in pGLUC-basic). Then cells were treated for 12 h with alpha-MSH, IBMX or db-cAMP. (B) Effect of puerarin and PT extract on MITF promoter activities. Luciferase activity was normalized by pGLuc-basic activity and the results were expressed as fold stimulation of luciferase activity from the unstimulated control. The cumulative (control value taken as one-fold in each case) data are presented as mean ± SE. *p < 0.05, **p < 0.01, ***p < 0.001

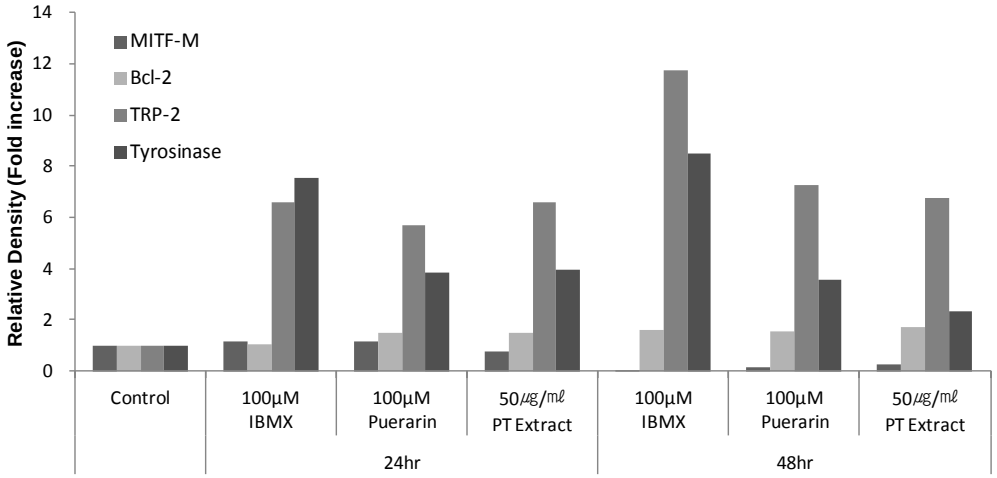
PT extract and puerarin stimulated the melanogenesis related protein expression

The expression levels of key regulators of melanin synthesis, MITF, tyrosinase and TRP-2 in cultured melanocytes were determined by western blot. 50uM puerarin and 50 μg/ml PT extract increased MITF-M, tyrosinase and TRP-2 protein expression in melan-a cell (Figure 7 A, B). MITF-M protein was expressed maximally at 24h while TRP-2 expression increased time-dependently until 48h. Furthermore, levels of an anti-apoptotic protein, Bcl-2 was also increased by the treatment of puerarin and PT extract. As previously shown, puerarin and PT extract increased the cAMP level in human cultured melanocytes (Figure 6A). cAMP is known as a key regulator for the expression of MITF through activation of PKA and CREB phosphorylation [12]. As shown in Figure 7 C and D, puerarin and PT extract increased the phosphorylation of CREB maximally at 2h treatment in cultured melanocyte while total CREB protein expression did not change. Taken together, our results suggest that puerarin and PT extract induce tyrosinase and TRP-2 expression through the up-regulation of MITF-M and CREB phosphorylation.

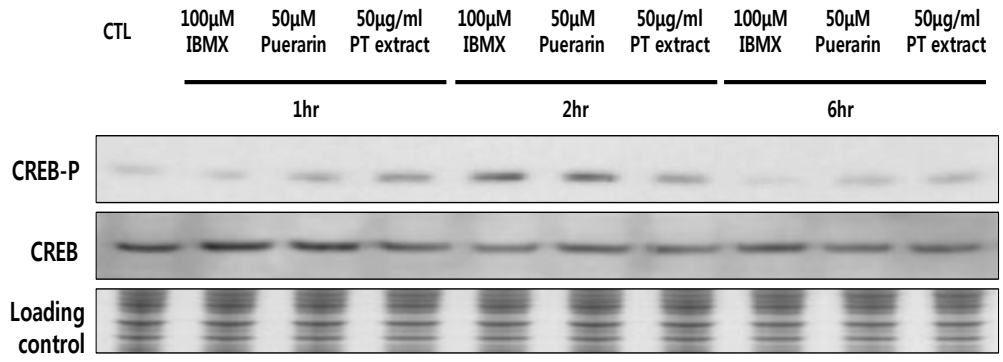
(A)


CTL
100μM IBMX
50μM Puerarin
50μg/ml PT extract
100μM IBMX
50μM Puerarin
50μg/ml PT extract
24hr
48hr
MITF-M
Bcl-2
TRP-2
Tyrosinase
Loading control

(B)


Relative Density (Fold increase)
14
12
10
8
6
4
2
0
MITF-M
Bcl-2
TRP-2
Tyrosinase
Control
100μM IBMX
100μM Puerarin
50㎍/㎖ PT Extract
100μM IBMX
100μM Puerarin
50㎍/㎖ PT Extract
24hr
48hr

(C)


CTL
100μM IBMX
50μM Puerarin
50μg/ml PT extract
100μM IBMX
50μM Puerarin
50μg/ml PT extract
100μM IBMX
50μM Puerarin
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1hr
2hr
6hr
CREB-P
CREB
Loading control

(D)

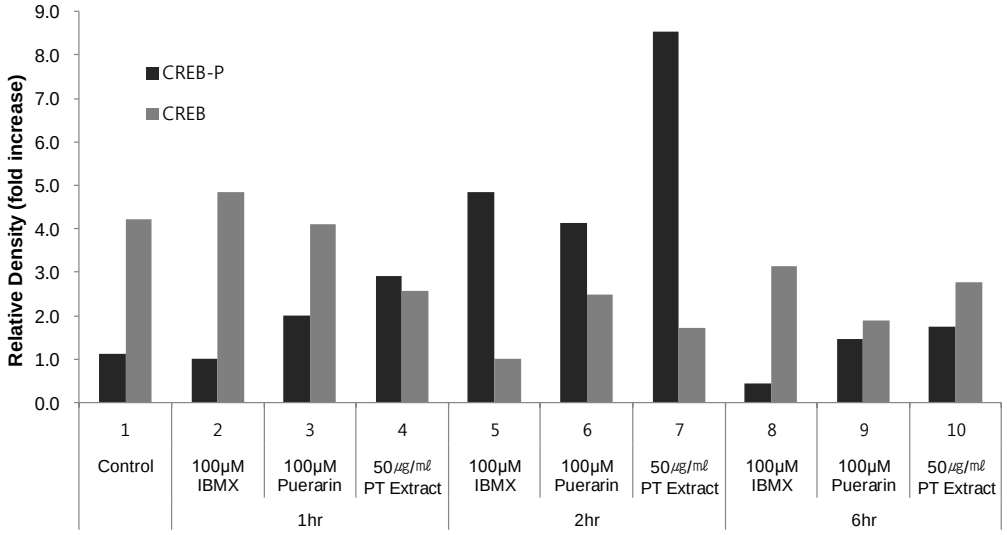
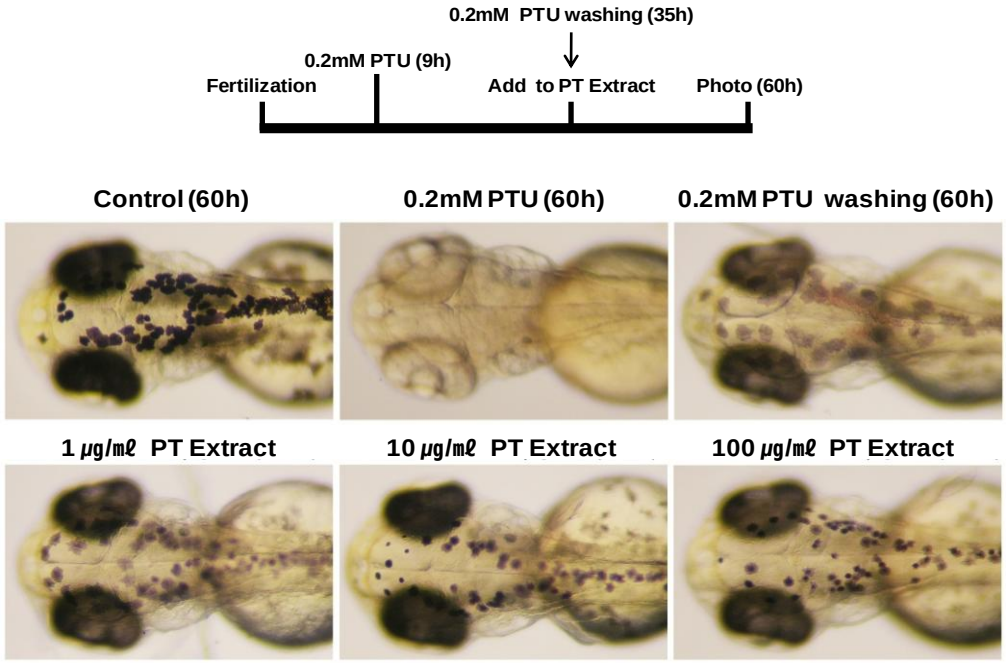

Relative Density (fold increase)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
CREB-P
CREB
1 Control
2 100μM IBMX
3 100μM Puerarin
4 50㎍/㎖ PT Extract
5 100μM IBMX
6 100μM Puerarin
7 50㎍/㎖ PT Extract
8 100μM IBMX
9 100μM Puerarin
10 50㎍/㎖ PT Extract
1hr
2hr
6hr

Figure 7. (A, B) Effect of puerarin and PT extract on the key regulating proteins in melanogenesis (MITF, tyrosinase, TRP-2) and melanocyte survival (Bcl-2). After incubation of melan-a cells with 100μM IBMX, 50μM puerarin and 50 ㎍/ml PT extract for 24, 48 h, the expressions of MITF-M, Bcl-2, TRP-2 and Tyrosinase proteins were presented as a western blot. (C, D) The effect of puerarin and PT extract on phosphorylation of CREB of melan-a cells. After incubation of melan-a cells with 100μM IBMX, 50μM puerarin and 50 ㎍/ml PT extract for 1, 2, 6 h, the phosphorylated CREB and total CREB are represented as a western blot.

PT extracts restored melanogenesis of zebrafish treated with 1-phenyl-2-thiourea

PT extracts were treated to synchronized embryos of zebrafish for evaluation of the effect on the pigmentation. The zebrafish embryos were treated with 0.2mM 1-phenyl-2-thiourea (PTU) for 26hr to generate transparent zebrafish and considered as a standard negative control. Zebra fishes were incubated in 3 concentration (1, 10, 100 μg/ml) of PT extract until 60h, then observed under the stereomicroscope. As shown in Figure 8, PT extracts were significantly increased the pigmentation of zebrafish embryos does-dependently. 0.2mM PTU strongly eliminated melanin in zebrafish embryos, and the pigments were partially restored after washing PTU. PT extracts stimulated the melanogenesis in PTU washed zebrafish, and these results suggest that PT extracts induced melanin synthesis *in vivo* model.

(A)


0.2mM PTU washing (35h)
0.2mM PTU (9h)
Fertilization
Add to PT Extract
Photo (60h)
Control (60h)
0.2mM PTU (60h)
0.2mM PTU washing (60h)
1 μg/mℓ PT Extract
10 μg/mℓ PT Extract
100 μg/mℓ PT Extract

(B)

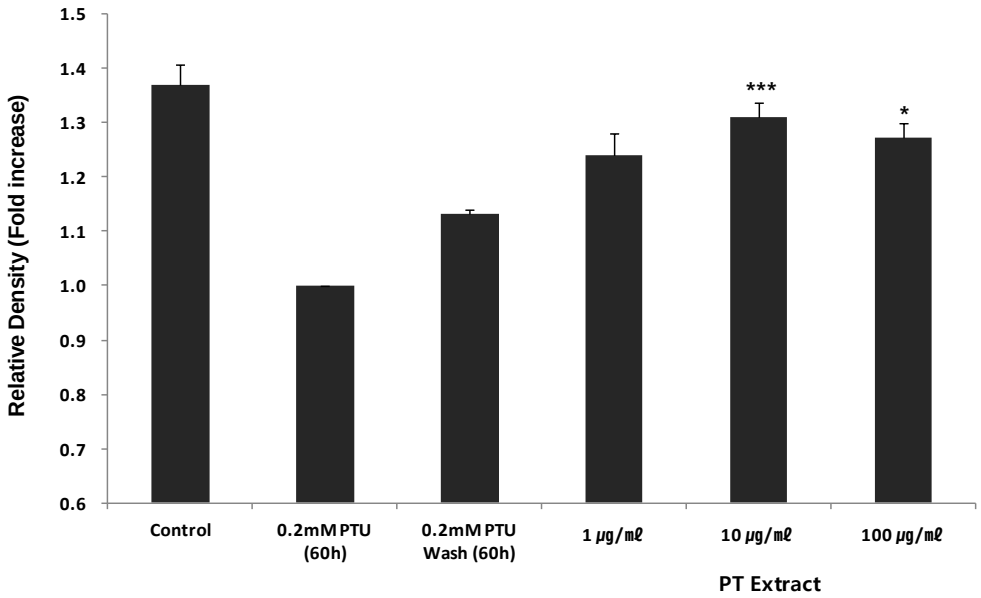

Relative Density (Fold increase)
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
***
*
Control
0.2mM PTU (60h)
0.2mM PTU Wash (60h)
1 μg/mℓ
10 μg/mℓ
100 μg/mℓ
PT Extract

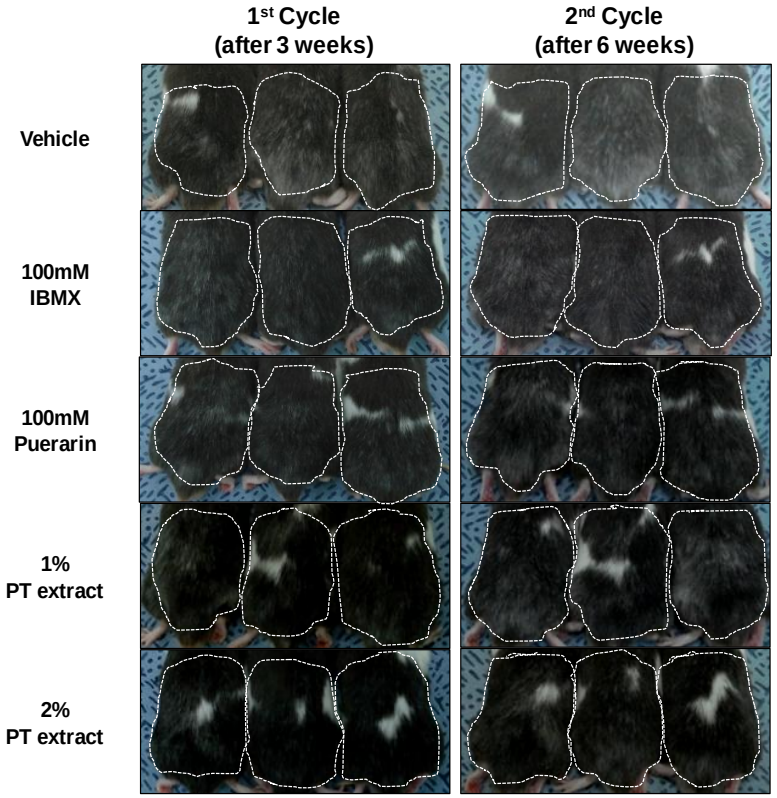
Figure 8. The Effects of PT extract on the pigmentation of zebrafish. Synchronized embryos were treated with test materials at the indicated concentrations. Test materials were dissolved in 0.1% DMSO, and were then added to the embryo medium. The effects on the pigmentation of zebrafish were observed under the stereomicroscope (A) and analyzed the melanin density (B) in the dorsal view of embryos at 60 h. After treatment with 0.2mM 1-phenyl-2-thiourea (PTU) for 26 h, zebra fishes were incubated at 3 concentrations (1, 10, 100 μg/ml) of PT extract until 60 h. *p < 0.05, **p < 0.01, ***p < 0.001 vs. 0.2mM PTU washing group

PT extracts and puerarin suppressed hair graying of MITF$^{vit/vit}$ mice

The pelages of the MITF$^{vit/vit}$ mice turned into gray after accelerating hair cycle by plucking. IBMX, a positive control, 1%, 2% PT extract and 100mM puerarin were topically applied on the dorsal skin for 3 weeks after plucking. As shown in Figure 9A left, the pelages of MITF$^{vit/vit}$ mice treated with IBMX, puerarin and PT extract were observed darker than vehicle treated group. After taking photographs, the hairs of the mice were replucked and applied again with IBMX, PT extract and puerarin for further 3 weeks. As shown in Figure 9A right, the newly developed dorsal hair appeared brighter than the generated hair by first plucking. The color of pelages of mice treated with IBMX, puerarin and PT extract were also darker than control group, especially 2% PT extract showed significantly dark color.

The amount of hair melanin in each group was measured as optical density (405 nm) per mg of hair after overnight digestion with protease. Figure 9B showed that IBMX, puerarin and PT extract significantly increased the melanin contents in regenerated hair compared to vehicle treated group. 2% of PT extract particularly showed significant increase in melanin contents repeatedly in first and second hair generation ($P<0.01$). These results suggest that PT extract and puerarin suppressed hair graying in murine model, and they could be used as a remedy for hair graying or vitiligo.

(A)


1st Cycle
(after 3 weeks)
2nd Cycle
(after 6 weeks)
Vehicle
100mM
IBMX
100mM
Puerarin
1%
PT extract
2%
PT extract

(B)

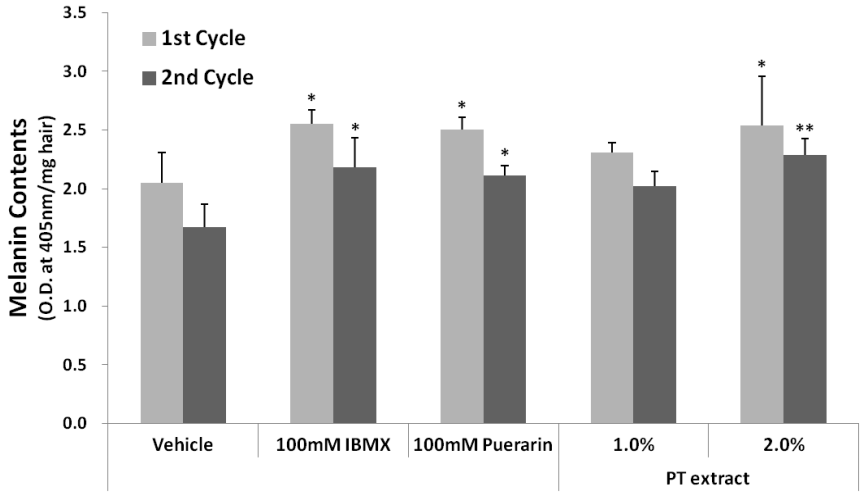

Melanin Contents
(O.D. at 405nm/mg hair)
1st Cycle
2nd Cycle
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Vehicle
100mM IBMX
100mM Puerarin
1.0%
2.0%
PT extract

Figure 9. The Effect of PT extract and puerarin on the restoration of hair pigmentation unit of *Mitf*$^{vit/vit}$ mice. (A) Effects of puerarin and PT extract on dorsal hair pigment restoration in the *Mitf*$^{vit/vit}$ mice depilated repeatedly. Acceleration of hair cycling was induced by the application of depilation twice, with a 3 week interval, upon the back skin of 12-week-old *Mitf*$^{vit/vit}$ mice. Test materials were dissolved in EtOH/Butylene glycol/Water(3/2/5), and were then topically administered to the back skin for 6 weeks. (B) Comparative hair melanin contents of the depilated dorsal area of *Mitf*$^{vit/vit}$ mice. The protease Esperase (Sigma, St Louis, MO) is used to measure the melanin content in hair. 10 mg of mouse hair is added to 1 mL of the reaction buffer, and hydrolysis reaction is carried out at 37℃ for 13 h, while shaking at 1,000 rpm. Melanin contents were represented as OD at 405nm of reaction solution per mg hair.

DISCUSSION

Hair graying is a typical sign of human aging. It occurs due to the functional failure of follicular melanocytes. Hair color is determined by the presence of melanin produced from the follicular melanocytes, and thus, the activation of melanin synthesis in melanocytes could enhance hair pigmentation. In this study, we demonstrated that the treatment of extract of *Pueraria thunbergiana* (PT extract) and its representative active compound, puerarin, stimulated melanogenesis in melanocytes and hair pigmentation via the up-regulation of the cAMP/MITF-M signaling pathway.

Firstly, we found that PT extract and puerarin increased the melanin contents in cultured melanocytes (Figure 5A). We hypothesized that PT extract and puerarin might regulate melanogenesis related factors, including cAMP, CREB and MITF-M. cAMP is a critical component of melanogenesis involved in both melanin synthesis and the transport of melanosomes. It activates PKA and CREB transcription factor, which leads to the production of MITF-M [12], a melanocyte-specific transcription factor crucial for melanocytes development and differentiation [14, 40]. Thus, we investigated the effect of PT extract and puerarin on cAMP levels in melanocytes at first, and they increased the cellular cAMP levels significantly compared to the non-treated control (Figure 5B). Furthermore, we found that PT extract and puerarin stimulated the phosphorylation of CREB using western blot (Figure 7C). These results suggest that PT extract and puerarin increase cAMP levels

and lead to the activation of CREB in cultured melanocytes.

To evaluate the effect on MITF, the down-streamed protein of CREB, we constructed reporter gene expression assays with MITF, tyrosinase and TRP-2 promoters inserted in pGluc-basic plasmid. Tyrosinase and tyrosinase-related protein-2 (TRP-2) which are involved in melanin biosynthesis, and are preferentially expressed in pigment cells. Tyrosinase is transcribed by MITF [41-43], and acts in the first process of melanogenesis from L-tyrosine. PT extract and puerarin significantly induced the MITF promoter activity (Figure 6), but did not work in tyrosinase and TRP-2 promoter (data not shown). We further examined the melanogenesis related protein expressions to verify the activities of those materials on MITF promoter activation. In contrast with the results of the promoter reporter assay, PT extract and puerarin increased the expressions of tyrosinase and TRP-2, as well as MITF-M. These results suggest that PT extract and puerarin enhance the expression of tyrosinase and TRP-2 via the elevation of MITF-M expression, and do not directly interact with their promoters. We also found that PT extract and puerarin enhanced the expression of Bcl-2, an anti-apoptotic protein responded by MITF-M.

From all of the results, we could conclude that PT extract and puerarin increase the melanin contents in melanocytes by potentiating the cAMP/MITF pathway (Figure 10).

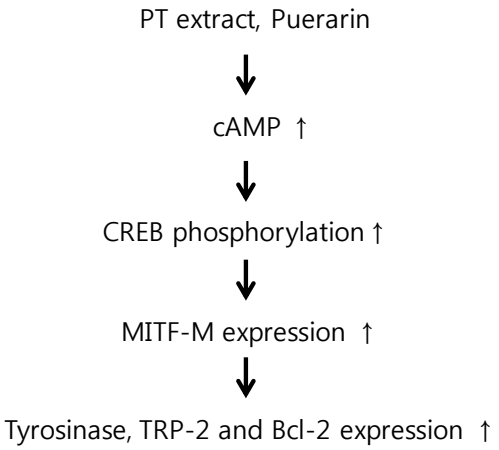


Figure 10. The mechanism of PT extract and puerarin on melanogenesis

We examined the embryos of zebrafish treated with 0.2mM PTU for investigating the effect on melanogenesis in tissue. PTU is a tyrosinase inhibitor used routinely to inhibit pigment production in zebrafish [44]. PTU treated zebrafish lost their pigmentation, and the melanin synthesis were effectively observed after treatment of PT extract and puerarin. This result suggests that PT extract and puerarin can stimulate the melanin synthesis in tissue through restoring the tyrosinase activity suppressed by PTU treatment.

We further examined the effect of those effective materials on hair darkening using the $MITF^{vit/vit}$ murine model. This microphthalmia-vitiligo mutant mouse are born with normal coat color, however the pelages loss their color and turn into gray after plucking hair at 12 weeks [39]. Thus this murine model was frequently used for development of new drugs for treating vitiligo. We applied PT extract and puerarin topically for 3 weeks after plucking the dorsal hair of 12 week-old $MITF^{vit/vit}$ mutant mice for evaluating the effect of anti-hair graying. Then we replucked the pelages and applied again for further 3 weeks to confirm the effect of hair darkening. The $MITF^{vit/vit}$ mice lost their coat color as they are aged, and the topical treatment of IBMX, puerarin and PT extract increased the hair melanin content significantly. In particular, 2% of PT extract showed a high effect of melanin maintenance in newly developed dorsal hair at second cycle.

These results of *in vivo* experiments using embryos of zebrafish and $MITF^{vit/vit}$ mice showed the possibility of PT extract as an anti-hair graying

agent. In conclusion, the PT extract and its active compound, puerarin, stimulate the melanogenesis via cAMP/ MITF-M signaling pathway *in vitro*, and they can prevent the follicular depigmentation by stimulating the melanin synthesis.

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국문 초록

백모 현상은 가장 대표적인 모발 노화 증상이며, 모낭 멜라닌형성세포가 이러한 백모 진행에서 많은 역할을 한다. 멜라닌형성 과정에서 중요한 전사인자인 MITF-M 은 모낭 멜라닌형성세포의 생존, 증식, 이동과 멜라닌 합성을 조절하는 것으로 알려졌다.

본 연구에서는 많은 천연물로부터 백모를 억제하는 새로운 물질을 탐색하고자 하였으며, 멜라닌형성세포와 멜라닌 합성과정에서 가장 중요한 신호전달 체계인 cAMP/MITF-M 경로를 조절하고자 하였다.

우선 많은 천연물로부터 melan-a 멜라닌형성세포가 생산하는 melanin 을 정량하고, 최초 신호전달 인자인 cAMP 함량을 측정하였다. 멜라닌 합성단계에서 주요한 전사인자와 합성효소의 조절을 알아보고자 각각의 프로모터 활성을 살펴 보았다. 또한, 이러한 cAMP/MITF-M 신호전달 체계에 관여하면서 멜라닌 합성과 멜라닌형성세포의 생존을 조절하는 단백질의 발현을 관찰하였다. 앞서의 세포수준의 평가와 더불어 최종적으로 zebrafish 와 자연변이 $MITF^{vit/vit}$ 마우스를 이용하여 평가를 진행하였다.

갈근 추출물과 대표적인 활성물질로 알려진 퓨에라린 화합물의 경우 세포내 cAMP 양을 증가시킴으로써 MITF 전사인자의 발현을 촉진하는 것을 확인하였다. 갈근 추출물과 퓨에라린은 멜라닌합성단계에서 중요한 역할을 담당하는 타이로신네이즈와 TRP-2 단백질과 멜라닌형성세포의 생존에 중요한 Bcl-2 단백질의 발현을 촉진하였다. 갈근 추출물과 퓨에라린을 zebrafish 에 처리했을때, 1-phenyl-2-thiourea 에 의해 탈색된 신체에서 다시 멜라닌형성을 촉진하는 것을 확인할 수 있었다. 다시 노화에 따라서 등쪽 털이 백색으로 변하는 백반증 마우스($MITF^{vit/vit}$ mice)를 제모하여 모발주기를 촉진시켜 백모를 가속화한 시킨 실험모델에서 갈근 추출물과 퓨에라린의 도포에 의해 백모화 현상을 저해 할 수 있었다.

이상의 실험결과를 통해서 갈근 추출물과 퓨에라린 화합물은 멜라닌형성세포의 cAMP/ MITF-M 신호전달 경로를 활성화시켜 멜라닌형성세포의 생존과 멜라닌합성을 촉진하여 백모나 백반증을 위한 새로운 치료의 대안으로 사용될 수 있을 것이다.

주요어: hair graying, MITF, Melanogenesis, *Pueraria thunbergiana*

학 번: 2005-31203