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The melanogenic effects and underlying mechanism of paeoniflorin in human melanocytes and vitiligo mice

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ABSTRACT

Vitiligo is a common depigmentary disease characterized as diagnosis simplicity and cure difficulty in view of the ambiguity of etiology, thus novel and effective treatments are urgently needed. Paeoniflorin, the major active compound extracted from the root of *Paeonia lactiflora Pall*, a traditional Chinese medicine, has been validated pharmacological properties such as antioxidant stress, a theory participating in the occurrence of vitiligo, but the effect on melanogenesis is still unclear. In this study, melanosythesis effect of paeoniflorin and the potential mechanism were evaluated. We found that treatment with paeoniflorin at the concentration of 10 μg/ml significantly increased melanin content and intracellular tyrosinase activity of human melanocytes, in accordance with the elevation of protein levels of microphthalmia-associated transcription factor (MITF), tyrosinase-related protein 1 (TRP-1). In addition, we also investigated that paeoniflorin promoted phosphorylation of cAMP-response element binding (CREB) and extracellular signal-regulated kinase (ERK) without affecting p38 and c-Jun N-terminal kinase (JNK). These results demonstrated that paeoniflorin had a synergistic effect on normal human melanocytes via ERK/CREB pathway with up-regulation of MITF and TRP-1, enhancing melanin synthesis. Meanwhile, the milder pathological changes in vitiligo mice treat with paeoniflorin also confirmed its potential in treating vitiligo. To sum up, we suggest that paeoniflorin may be a potential medicine of vitiligo treatment in clinical.

1. Introduction

Vitiligo is the most common pigmentary disorder around the world, perplexing approximately 0.5%–2% population for both sexes and all races [1]. Characterized as complete patchy depigmentation, be it apparently visible white patches or maculae which may affect the appearance negatively, vitiligo has brought great suffering to the patients, especially girls and women who are wild about beauty. Up to now the etiology and pathogenesis of vitiligo still remain ambiguous, one of the hypotheses backed up by many studies is the theory of oxidative stress, that the oxidative stress takes a vital part in the occurrence and development of vitiligo and may destroy melanocytes. [2,3]. On all accounts, the core event is the scarcity of functioning melanocytes and melanin which directly lead to the depigmentation (Ezzedine, Eleftheriadou [4].

Under normal circumstance, melanocytes mature with growth and produce melanin adequately to maintain even color of skin, which is initiated by tyrosinase-related protein 1 (TRP-1) and tyrosinase-related protein 2 (TRP-2), the two enzymes prerequisite in melanin production can be activated by microphthalmia-associated transcription factor (MITF)(Vachtenheim and Borovansky [5]. Melanin synthesis is documented to be controlled by diverse intracellular signaling pathways, such as α-MSH/cAMP-dependent signaling pathway and MAPK signaling pathway. MAPK cascade affecting melanogenesis is increasingly understood, whose family members including ERK, JNK, p38 and so on. The phosphorylation of p38 activates MITF and downstream proteins, ultimately stimulating melanin synthesis [6,7]. On the contrary, the JNK activation exerts negative regulatory role in melanin production,

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directly leading to the degradation of MITF [8]. Extracellular-signal-regulated kinase (ERK), on the one hand, can trigger MITF gene expression by means of the activation of cAMP-response element binding (CREB) protein, which binds to MITF promoter region to upregulate melanin content, on the other hand, the elevated expression of p-ERK is associated with degradation of MITF [9].

To remedy vitiligo, treatments often aim at repigmentation of the decolorizing areas, which can be implemented by retrieving the damaged melanocytes and melanin. Therapies of vitiligo are classified as Medical treatment and Surgical techniques [10], both with merits and values. The former, encompassing Corticosteroids, Calcineurin Inhibitors, Immunosuppressive agents and etc., is prior and advantageous for its noninvasive nature and fargoing applicability, no matter progressive cases or stable, which in turn make it acceptable for substantial patients. However, the therapeutic regimens available still leave much to be desired, it is imperative to find some novel and effective treatments.

Paeoniflorin, the active compound extracted from the root of *Paeonia lactiflora Pall*, a kind of traditional Chinese medicine being employed in Chinese herbal compound prescriptions in vitiligo treatment, has been confirmed to possess numerous pharmacological properties including antioxidant stress [11], whereas there are no definite proof of paeoniflorin affecting melanocytic functions. Herein, we speculated that paeoniflorin have the ability to stimulate melanogenesis and investigated the underlying mechanism, the role of paeoniflorin in mice vitiligo models was studied as well.

2. Materials and methods

2.1. Materials

Paeoniflorin (purity > 98%, HPLC) was purchased from Solarbio (Beijing, China) and stored in deionized water at the concentration of 10 mg/ml for cell experiment. Dispase II, growth medium M-254, human melanocyte growth supplement-2 (HMGS-2), and L-DOPA (L-3, 4-dihydroxyphenylalanine) were purchased from Sigma Chemical Co (St. Louis, MO, USA). Primary antibodies against ERK, phospho-ERK (p-ERK), JNK, phospho-JNK (p-JNK), p38, phospho-p38 (p-p38), CREB, phospho-CREB (p-CREB) and actin were purchased from Cell signaling technology (Boston, USA). Primary antibodies against MITF, TRP-1, GAPDH were purchased from Abcam (Cambridge, MA, USA). Whereas Goat anti-rabbit and Goat anti-mouse horseradish peroxidase conjugates were purchased from BioRad Laboratories (Calif, USA).

3. Method

3.1.1. Isolation and culture of melanocytes

Detailed description of isolation and culture of human melanocytes has been documented previously [12]. Briefly, the surgically excised human foreskin sample was submerged in iodine solution and 75% ethanol solution successively in case of contamination, then washed in PBS with penicillin (500 IU/ml) and streptomycin (500 mg/ml) for a few seconds. By using ophthalmic scissors and tweezes, after that, the subcutaneous fat and connective tissue was cleared as much as possible. The sample cut into pieces was put epidermis-side-down in Dispase II (2 mg/ml) in 4 °C refrigerator. After overstaying in 4 °C, the epidermis was gently peeled away from the dermis and digested in 0.25% trypsin/EDTA solution in 37 °C for approximately 10 min. The sample epidermis was dispersed into cell suspension and centrifuged at 1000 rpm for 3 min. Supernatant removed, the black cell mass was resuspended in the M-254 supplemented with 1% HMGS-2. Finally, the extracted cells were plated in culture flasks and incubated in a humidified, 37 °C, 5% CO_2 incubator. Change the media after 24 h and thereafter every 3 days.

3.1.2. Melanocytes identification

To determine the phenotypes of the isolated melanocytes in vitro, cells were examed by L-3, 4-dihydroxyphenylalanine (L-DOPA) staining. A pre-setting cell climbing slice was washed with PBS and fixed with 4% paraformaldehyde before incubated in L-DOPA staining solution (1 g/l) at 37 °C for 4 h. The slice was then washed with PBS for another 5 min to ensure that the residual L-DOPA was cleared up. The morphology of melanocytes was observed under the transmission electron microscope and recorded.

3.1.3. Cell proliferation and viability assay

We assessed the effect of paeoniflorin on melanocytes proliferation and viability by CCK-8 assay. Melanocytes were seeded into 96-well plates at density of 1×10^4/well for 48 h, and then treated with different concentrations of paeoniflorin, ranging from 2.5 to 1000 μg/ml for another 48 h. Using automicroplate reader (Bio-Rad, USA), afterwards , the absorbance rate was measured at 450 nm tautologically and the data was collected until the OD value of the untreated group approached 1.

3.1.4. Melanin content assay

Melanin content of melanocyte is measured by the method previously discussed [13]. Prepared human melanocytes were seeded into T-25 flasks at a density of 1×10^5 cells/ml for overnight incubation first and then were exposed to different concentration of paeoniflorin (0, 5, 10 μg/ml) for another 24 h. Cell numbers were counted respectively before cell deposits presenting different drug levels were obtained by enzymatic dissociation method. Afterwards, the cell pellets were suspended with PBS and dissolved in 100 μl of 1 M NaOH after finial centrifugation (1000 rpm for 5 min). The samples were then heated at 80 °C for 1 h to solubilize the melanin pigment and then transferred to 96-well plates, the absorbance of solution was measured at 405 nm. Final results were expressed as a percentage of the control - The group that was not treated with paeoniflorin.

3.1.5. Celluar tyrosinase activity assay

Analysis of intracelluar tyrosinase activity was performed by measuring the rate of oxidation of L-DOPA, and the steps were proceeded as previously discussed with slight modification [14]. Exponentially growing human melanocytes were treated with paeoniflorin in T-25 flasks at a density of 5×10^5 cells/ml. After a 24 h incubation, cells were lysed and clarified by centrifugation to obtain a supernatant, then protein concentrations were determined, and 100 μl of each lysate was shifted in a 96-well plate. L-DOPA (2 mg/ml) solution was prepared and the enzymatic assay was initiated by the addition of 40 μl L-DOPA solution and then incubated at 37 °C. Measure the absorbance at 475 nm every 10 min until the reaction was terminated at 1.5 h. The result was expressed as the percentage of the controls. All experiments were repeated at least 3 times with similar results.

3.1.6. Western blotting

For western blot analysis, cells were lysed to acquire proteins, followed by sonication on ice and centrifugation at 12,000 rpm for 30 min. Protein concentrations of each sample were determined using the Bradford method with BSA as the standard. 20 μg of total protein were separated in each lane of 10% SDS–PAGE gels, and then were transferred to a PVDF membrane. The blots were blocked with 5% BSA in TBST (Tris-buffered saline with 0.1% Tween-20) at room temperature for 2 h, the membranes then were transferred to primary antibodies for 24 h, followed by incubation with secondary antibody for 1 h. Finally the proteins were detected using enhanced chemiluminescence (ECL) reagents (Thermo Scientific), and quantified by the Quantity ONE (Bio-Rad, Hercules, CA, USA) software. The GAPDH and Actin antibody was used to monitor protein loading in each lane.

3.1.7. Models of vitiligo in mice

Vitiligo model mice were induced with 40% monobenzone (4-benzyloxyphenol, Sigma). As previously described [15], monobenzone cream was applied on 4-week-old female SPF C57BL/6 mice topically for 8 weeks and further observation was continued until 12 weeks. The control group were treated with petrolatum simultaneously. The mice were purchased from Animal Center of Chinese Academy of Sciences, Shanghai, China. The protocol for animal care and use conformed to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and was approved by the Animal Care and Use Committee of Wenzhou Medical University.

3.1.8. Experimental animal design

The experimental mice were randomly consisted of three groups of 10 mice: control group (CL), normal mice received no treatment; vitiligo model mice with paeoniflorin treatment group, mice in this group were injected with 60 mg/kg paeoniflorin in 20% propanediol via intraperitoneal injection [16]; vitiligo model mice administrated same volume of saline. All the treatment lasted for 10 days. Mice were feed under a constant temperature of 20 ± 2C°, a relative humidity of 50% ± 10%, and a 12 h light/dark cycle, with food and water available.

3.1.9. Histopathologic analysis

Depigmentary skin samples distant from the monobenzone administration site were obtained and tissue wax blocks of different groups were prepared for histological analysis. 5 mm mice skin sections were subjected to routine Hematoxylin and Eosin Staining, beyond that Masson-Fontana staining, a staining special for melanin, was also performed to clearly demonstrate the histopathologic changes and all the stainings were evaluated microscopically.

3.1.10. Statistical analysis

All data was expressed by the mean ± standard deviation (SD), further statistical analysis was performed using GraphPad Prism 7 and statistical significance was calculated by Student's *t*-test. The value of $p < .05$ (*) was considered to be significant.

4. Results

4.1. Morphology of human melanocytes and L-DOPA staining result

The extracted normal human melanocytes exhibited bipolar and dentritic morphology, which could be observed in Fig. 1b and c. Melanocytes were clearly defined and showed a distinct black under L-DOPA staining (Fig. 1e).

4.2. Effect of paeoniflorin on proliferation and viability of human melanocytes

The effect of paeoniflorin on human melanocytes viability and proliferation was assessed at various concentrations (2.5–1000 μg/ml) for 48 h using CCK-8 assay. Cells without treatment were kept as control, and the result of the control group was assigned 100%. As shown in Fig. 2a, obvious melanocyte proliferation was observed at the concentrations of 5 and 10 μg/ml ($p < .05$), and the group treated with 10 μg/ml paeoniflorin showed the highest proliferation rate. Other concentrations had no significant effect, neither proliferation nor inhibition, until the concentration reached 1 mg/ml when cell viability decreased. Based on the results, the experiment of melanin content was performed by treating cells with 5 μg/ml and 10 μg/ml paeoniflorin.

4.3. Paeoniflorin promotes melanin content of human melanocytes

We evaluated the level of melanin produced in human melanocytes after treating with paeoniflorin at 5 and 10 μg/ml for 48 h, meanwhile 50 μM 8-MOP group was assigned as positive control [17]. Melanin production was considerably boosted in the group of 10 μg/ml paeoniflorin ($p < .01$), which was similar to those in positive control group (Fig. 2b). Results suggested that 10 μg/ml paeoniflorin possess stimulating effects on melanin synthesis and thus which concentration was selected for following cellular tyrosinase activity and protein expression assessment.

4.4. Paeoniflorin upregulates cellular tyrosinase activity of human melanocytes

Tyrosinase is an essential rate-limiting enzyme involved in the key steps of melanin generation, it represents cells' ability to synthesize melanin to some extent. As portrayed in Fig. 2c, Paeoniflorin at the concentrations of 10 μg/ml, resembling positive control group, could largely accelerate cellular tyrosinase activity compared to control groups ($p < .05$).

4.5. Paeoniflorin affects protein expression of TRP-1 and MITF

As crucial regulating protein, the protein expression of TRP-1 and MITF influence melanin synthesis. Western blotting was carried out to measure protein expression of TRP-1 and MITF. The results indicated that addition of Paeoniflorin (10 μg/ml) for 48 h would increase TRP-1 and MITF expression levels compared to the controls ($p < .01$).

4.6. Effect of paeoniflorin on mitogen-activated protein kinase pathways

Mitogen-activated protein kinase pathways have been extensively studied in intracellular signaling pathways involved melanosynthesis. According to western bloting analysis results (Fig. 3a, 3b, 3c), paeoniflorin promoted phosphorylation of ERK and CREB whereas p38 and JNK were not influenced.

4.7. The treatment effect of paeoniflorin in vitiligo mice model

White spots were observed in the mice with monobenzone as shown in Fig. 3d. Generally after 8 weeks of topical application of monobenzone cream, white patches appeared in the drug-exposed area, and gradually expanded to distant sites such as trunk, ears, and/or tail, isolated or diffused, when drug was withdrawn.

10-day-treatment later, the discolored skin sections away from the monobenzone application site were obtained. As shown in Fig. 3e, the number of hair follicles in the paeoniflorin group was obviously higher than that in the vitiligo group, and the distribution of melanin surrounded was distinct as well. Masson-Fontana staining results consistent with that of HE staining, showed that the distribution of hair follicles and melanin was serried in paeoniflorin group and normal mice group.

5. Discussion

Vitiligo is a chronic, acquired cutaneous depigmentary disease in the skin and mucosa. The exact etiology and pathogenesis still remain blurry although varied theories have been put up forward [18]. Anyhow, deficient functioning melanocytes and lacking melanin directly result in the depigmentation. Medical treatment occupies the mainstream in therapeutic regimen of vitiligo, including Glucocorticoids, Calcineurin inhibitors, Phototherapy and etc. [10]. On top of those standard treatment options, Traditional Chinese Medicine (TCM) as an indispensable supplement has received increasing acceptance with certain clinical application value and prompt further investigation [19]. Numerous herbal compound prescriptions are intended to treat vitiligo in TCM, among which *Paeonia lactiflora Pall* namely shaoyao or baishao in Chinese is an active part. Paeoniflorin, the active compound extracted from the root of *Paeonia lactiflora Pall*, has been confirmed to

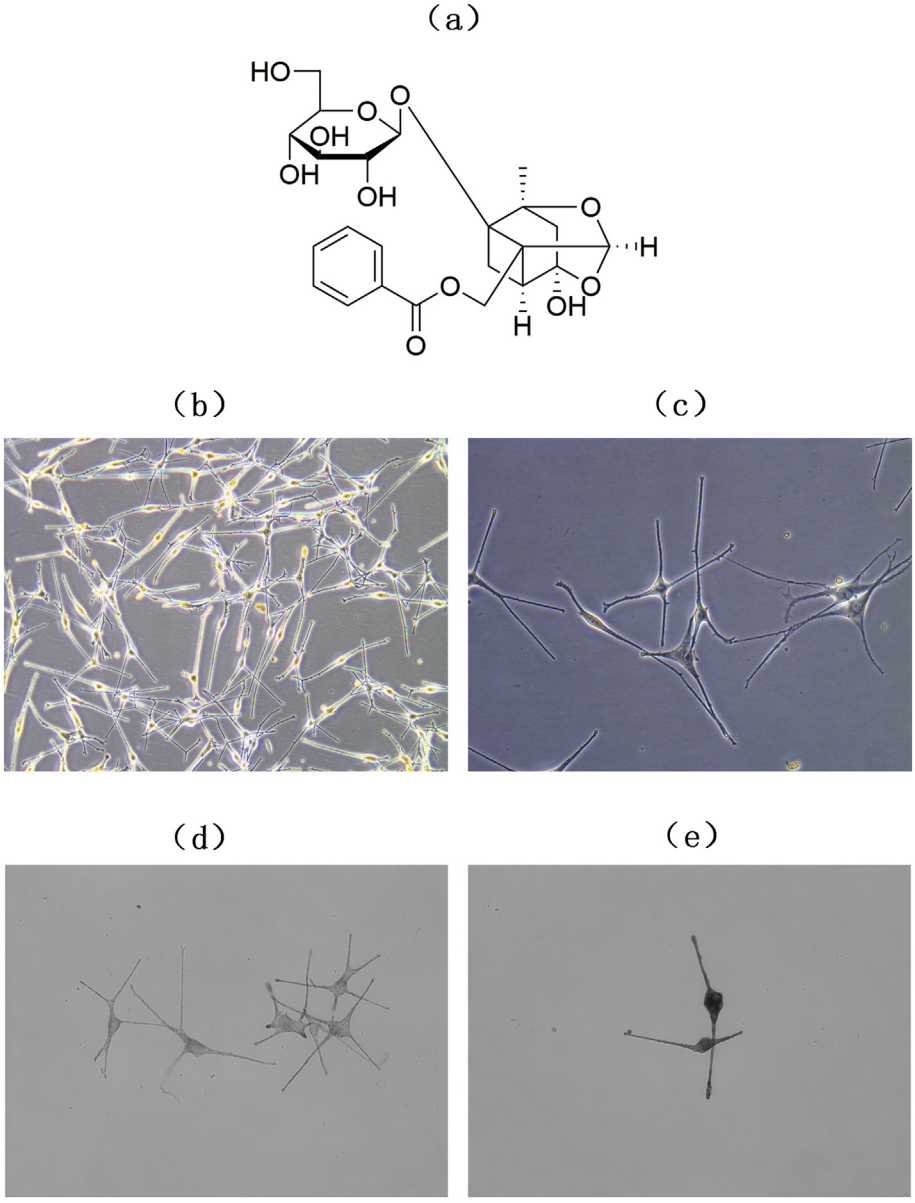

Fig. 1. (a) Molecular structure of paeoniflorin; (b), (c) Primary melanocytes extracted from human foreskins under 10-fold and 40-fold microscope; (d) Melanocytes before 3, 4-dihydroxyphenylalanine staining; (e) Melanocytes after 3, 4-dihydroxyphenylalanine staining for identification.

possess pharmacological properties such as antioxidant, oxidative stress theory being one of the pathogenesis theories in vitiligo, anti-inflammatory effect and so on [20–22]. Thus we speculated paeoniflorin may affect melanin synthesis and expression of melanin-related proteins positively, which was proved via a series of relevant experiments. MITF is a master regulator of melanogenesis and can upregulate melanogenic genes (Tyrosinase, TRP-1 and TRP-2) [23]. Tyrosinase is the rate-limiting enzyme in melanogenesis [24], catalyzing the hydroxylation of tyrosine and oxidizing downstream DOPA into DOPA quinone, and spontaneously melanin get synthesized. TRP-1 and TRP-2 function in the biosynthesis of melanin downstream of TYR [25], whose activity was performed by measuring the rate of oxidation of L-DOPA. Recent research have shown that Dopa oxidation assay based on Mushroom-Tyrosinase (mh-Tyr) to exam tyrosinase activity is conflicting in some dihydrochalcones related enzyme inhibition experiments [26]. Our study focus on increasing tyrosinase activity, the kind of error existed in inhibition experiments can be neglected. What's more, Paeoniflorin is a monoterpene glycoside compound without phenols structures which may interfere mh-Tyr activity. Concordant with our speculation, our results revealed that paeoniflorin could increase the melanin content and tyrosine activity in human melanocytes at the concentration of 10 μg/ml along with the ascending protein level of TRP-1 and MITF.

MAPK signaling pathway as one of the diverse intracellular signaling pathways governing melanogenesis have been widely studied. As shown in Fig. 4, generally phosphorylation of p38 activates melanin biosynthesis, the JNK activation is related to degradation of MITF and restrain the melanogenesis, and the role of ERK can be either activatory or inhibitory. Phosphorylated ERK can increase melanin pigmentation though there is an association with the degradation of MITF to some extent [9]. According to.

previous literatures, paeoniflorin exerts its pharmacological effects largely through the MAPK pathway [27–29]. Similarly, we inferred that the melanogenesis capacity of paeoniflorin also can be regulated by MAPK pathway, and consequently the role of MAPK signaling pathways was assessed. Results demonstrated that 10 μg/ml paeoniflorin increased the phosphorylated ERK and CREB without influencing phosphorylation of p38 and JNK in human melanocytes, the incentive effect of which is able to boost expression of melanin-related proteins (MITF,

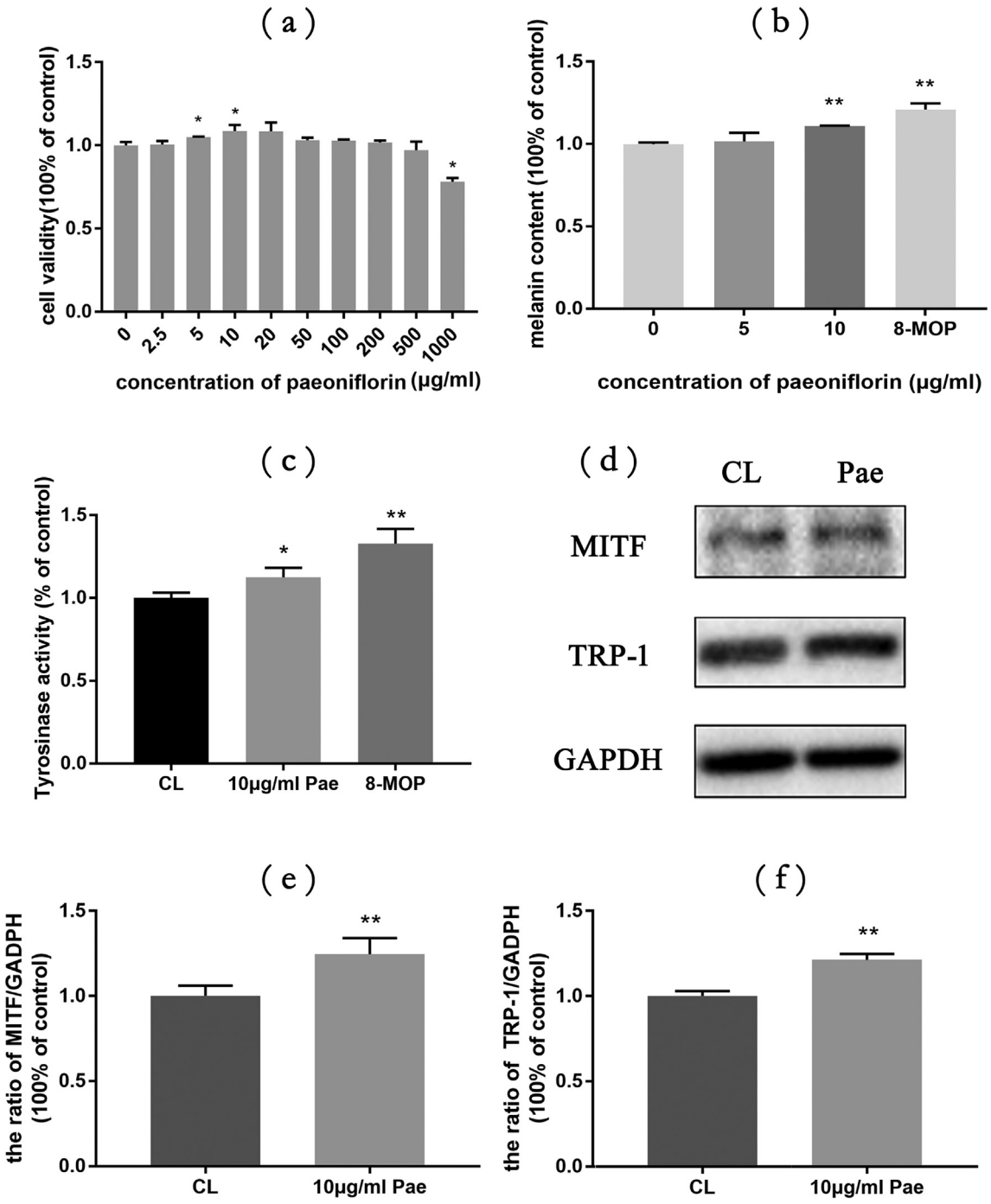

Fig. 2. (a) CCK-8 assay results indicated the effects on proliferation and viability of human melanocytes under our treatment with various concentrations of paeoniflorin and were presented in the format of mean ± standard deviation. *$p < .05$ vs. the control, **$p < .01$ vs. the control. (b) Effects of paeoniflorin on melanin content in human melanocytes, therein 50 μM 8-MOP was assigned as positive control. Melanin production was enhanced with the treatment of 10 μg/ml Paeoniflorin (**$p < .01$, vs. the control). (c) Analysis of tyrosinase activity was performed by measuring the rate of oxidation of L-DOPA. Paeoniflorin of 10 μg/ml promoted celluar tyrosinase activity in human melanocytes, was similar to 50 μM 8-MOP group (positive control), *$p < .05$ vs. the control. (d, e, f) Effects of 10 μg/ml paeoniflorin on the protein levels of TRP-1 and MITF in human melanocytes, which was evaluated by Western blot analyses. Values represented mean ± SD of three independent experiments; **$p < .01$ vs control was regarded as statistically significant. 8-MOP: 8-methoxypsoralen; MITF: microphthalmia-associated transcription factor; TRP-1: tyrosinase-related protein 1; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

TRP-1) and melanin content. We concluded that paeoniflorin exerts melanogenic function in normal human melanocytes by activating CREB and phosphorylating ERK.

To further support above results and to verify paeoniflorin interfering vitiligo repigmentation, we administrated paeoniflorin via intraperitoneal injection on vitiligo model mice, which is rarely operated. Anteriorly, an original mouse model of vitiligo was developed by topically applying the skin depigmenting agent monobenzone and the histological examination indicated loss of hair follicles and melanin [15]. Nevertheless in normal mice skin, the dorsal epidermis is very thin with numerous hair follicles surrounded by dense melanin. In our research, likewise, white patches distant from the administration site were founded which gradually expanded and were tended to stabilize within 1–4 weeks after discontinuation of the medication. Both gross

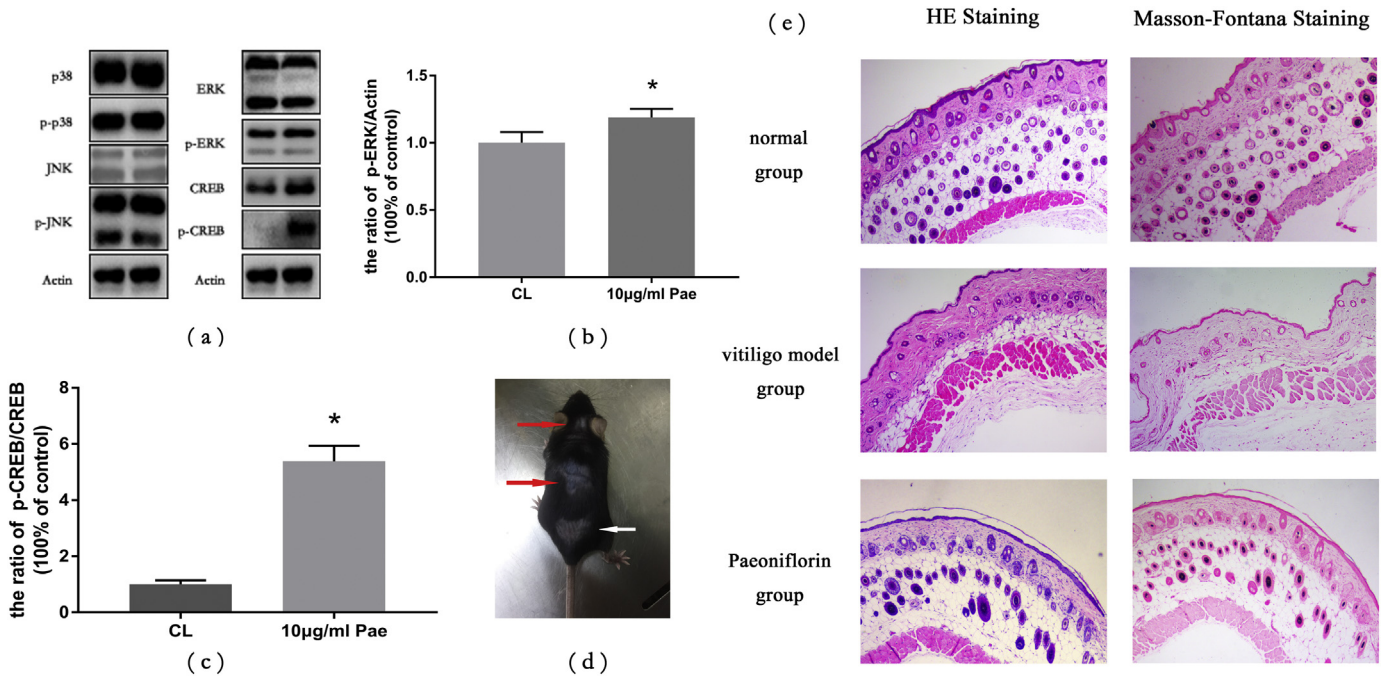

Fig. 3. (a, b, c) Effects of paeoniflorin on p38, JNK, ERK, CREB and counterpart phosphorylation protein expression. Densities were quantified with Image J software. Values represented mean ± SD of three independent experiments; *$p < .05$ vs control was regarded as statistically significant. (d) Vitiligo mouse models. White arrow: site with monobenzone cream application; Red arrow: distant white patches without applying monobenzone. (e) Staining results. The first line is HE Staining, and the other line is Masson-Fontana Staining. Both results are similar, the number of hair follicles in the paeoniflorin treat group was significantly higher than that in the vitiligo model group, accompanied by the increased distribution of melanin around. ERK: extracellular signal-regulated kinase; JNK: c-Jun N-terminal kinase; CREB: cAMP-response element binding protein; HE Staining: Hematoxylin-eosin staining.

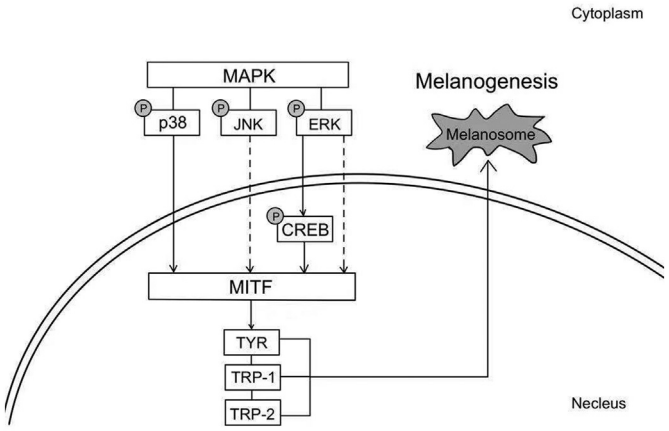

Fig. 4. The role of MAPK pathway regulating melanogenesis. The full lines means activation, whereas the dotted lines represent inhibition. The schematic referred to the cited article [8].

and pathological results illustrated that the hair follicles decreased greatly in model group, accompanied by decent decrease in peripheral melanin. After treatment with paeoniflorin, the vitiligo model mice showed milder pathological changes that hair follicles as well as melanin surrounded added.

6. Conclusion

Altogether, we came to the conclusion that paeoniflorin can affect the cell proliferation actively and increase the melanin biosynthesis of human melanocytes via activating CREB and ERK at the concentration of 10 μg/ml. Paeoniflorin administration on the vitiligo model mice, in addition, also exhibited certain therapeutic effect. Believed through our study, we validated the medicinal value of paeoniflorin in treating vitiligo, and advised the potential therapeutic agent acquire further research.

Declaration of Competing Interest

The authors have declared that there is no conflict of interests.

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References

[1] A. Taieb, M. Picardo, V. Members, The definition and assessment of vitiligo: a consensus report of the Vitiligo European Task Force, Pigment Cell Res. 20 (1) (2007) 27–35.
[2] H. Xie, et al., Vitiligo: how do oxidative stress-induced autoantigens trigger autoimmunity? J. Dermatol. Sci. 81 (1) (2016) 3–9.
[3] L. Denat, et al., Melanocytes as instigators and victims of oxidative stress, J. Invest. Dermatol. 134 (6) (2014) 1512–1518.
[4] K. Ezzedine, et al., Vitiligo, Lancet 386 (9988) (2015) 74–84.
[5] J. Vachtenheim, J. Borovansky, "Transcription physiology" of pigment formation in melanocytes: central role of MITF, Exp. Dermatol. 19 (7) (2010) 617–627.
[6] B. Saha, et al., Activation of the Mitf promoter by lipid-stimulated activation of p38-stress signalling to CREB, Pigment Cell Res. 19 (6) (2006) 595–605.
[7] Y. Mizutani, et al., A single UVB exposure increases the expression of functional KIT in human melanocytes by up-regulating MITF expression through the phosphorylation of p38/CREB, Arch. Dermatol. Res. 302 (4) (2010) 283–294.
[8] T. Pillaiyar, M. Manickam, S.H. Jung, Downregulation of melanogenesis: drug discovery and therapeutic options, Drug Discov. Today 22 (2) (2017) 282–298.
[9] G. Imokawa, K. Ishida, Inhibitors of intracellular signaling pathways that lead to stimulated epidermal pigmentation: perspective of anti-pigmenting agents, Int. J. Mol. Sci. 15 (5) (2014) 8293–8315.
[10] M. Rodrigues, et al., Current and emerging treatments for vitiligo, J. Am. Acad.

Dermatol. 77 (1) (2017) 17–29.
[11] S. Parker, et al., A pharmacological review of bioactive constituents of Paeonia lactiflora Pallas and Paeonia veitchii lynch, Phytother. Res. 30 (9) (2016) 1445–1473.
[12] L.S. Godwin, et al., Isolation, culture, and transfection of melanocytes, Curr. Protoc. Cell Biol. 63 (2014) 1 8 1–20.
[13] P. Xu, et al., Effects of aqueous extracts of Ecliptae herba, Polygoni multiflori radix praeparata and Rehmanniae radix praeparata on melanogenesis and the migration of human melanocytes, J. Ethnopharmacol. 195 (2017) 89–95.
[14] J.M. Hwang, et al., Inhibitory effect of liposome-encapsulated anthocyanin on melanogenesis in human melanocytes, Pharm. Biol. 51 (8) (2013) 941–947.
[15] Y. Zhu, S. Wang, A. Xu, A mouse model of vitiligo induced by monobenzone, Exp. Dermatol. 22 (7) (2013) 499–501.
[16] X. Zhong, et al., Paeoniflorin ameliorates chronic stress-induced depression-like behaviors and neuronal damages in rats via activation of the ERK-CREB pathway, Front Psychiatry 9 (2018) 772.
[17] L. Yin, et al., A novel psoralen derivative-MPFC enhances melanogenesis via activation of p38 MAPK and PKA signaling pathways in B16 cells, Int. J. Mol. Med. 41 (6) (2018) 3727–3735.
[18] A. Alikhan, et al., Vitiligo: a comprehensive overview part I. Introduction, epidemiology, quality of life, diagnosis, differential diagnosis, associations, histopathology, etiology, and work-up, J. Am. Acad. Dermatol. 65 (3) (2011) 473–491.
[19] Y.J. Chen, et al., Oral Chinese herbal medicine in combination with phototherapy for vitiligo: a systematic review and meta-analysis of randomized controlled trials, Compl. Ther. Med. 26 (2016) 21–27.
[20] J. Tu, et al., The regulatory effects of paeoniflorin and its derivative paeoniflorin-6'-O-benzene Sulfonate CP-25 on inflammation and immune diseases, Front. Pharmacol. 10 (2019) 57.
[21] S. Song, et al., Protective effects of paeoniflorin against AOPP-induced oxidative injury in HUVECs by blocking the ROS-HIF-1alpha/VEGF pathway, Phytomedicine 34 (2017) 115–126.
[22] X.H. Ma, et al., Neuroprotective effect of paeoniflorin on okadaic acid-induced tau hyperphosphorylation via calpain/Akt/GSK-3beta pathway in SH-SY5Y cells, Brain Res. 1690 (2018) 1–11.
[23] A. Kawakami, D.E. Fisher, The master role of microphthalmia-associated transcription factor in melanocyte and melanoma biology, Lab. Investig. 97 (6) (2017) 649–656.
[24] S.H. Park, et al., Terrein: a new melanogenesis inhibitor and its mechanism, Cell. Mol. Life Sci. 61 (22) (2004) 2878–2885.
[25] H. Nakagawa, Comparison of the efficacy and safety of 0.1% tacrolimus ointment with topical corticosteroids in adult patients with atopic dermatitis: review of randomised, double-blind clinical studies conducted in Japan, Clin. Drug Investig. 26 (5) (2006) 235–246.
[26] F. Mayr, et al., Mushroom Tyrosinase-based enzyme inhibition assays are not suitable for bioactivity-guided fractionation of extracts, J. Nat. Prod. 82 (1) (2019) 136–147.
[27] S. Ye, et al., Thrombosis recanalization by paeoniflorin through the upregulation of urokinasetype plasminogen activator via the MAPK signaling pathway, Mol. Med. Rep. 13 (6) (2016) 4593–4598.
[28] J. Zhang, et al., Paeoniflorin inhibits proliferation of endometrial cancer cells via activating MAPK and NF-kappaB signaling pathways, Exp. Ther. Med. 14 (6) (2017) 5445–5451.
[29] J. Yu, et al., Paeoniflorin suppressed IL-22 via p38 MAPK pathway and exerts antipsoriatic effect, Life Sci. 180 (2017) 17–22.