

Hydrolyzed Conchiolin Protein Inhibits Dendrite Formation of Melanocytes Via Wnt/ β -catenin Signaling Pathway

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Synopsis

Soluble pearl extracts present the potential in inhibiting melanin synthesis in B16 melanoma cells. However, the role of hydrolyzed conchiolin protein (HCP), the main protein ingredient in soluble pearl extracts, in skin lightening remains unclear. In this study, we observed that HCP effectively inhibited endothelin-1 (ET-1) induced melanocyte dendrite formation in both PIG1 and MelanA cells, demonstrating its excellent antagonistic properties against ET-1 in melanosome transfer. HCP also exhibits the capability to reduce ET-1-induced melanin secretion in MelanA cells. HCP also significantly suppressed the upregulation of microphthalmia-associated transcription factor (MITF), tyrosinase (TYR), Rab27a, and Rac1 gene expression. Additionally, HCP treatments led to a notable reduction in the protein expression levels of p-GSK-3 β , β -catenin, as well as the nuclear translocation of β -catenin. Importantly, HCP exhibited low toxicity towards MelanA and PIG1 cells. Our results indicate that HCP can effectively suppress melanin transfer in melanocytes by antagonizing ET-1 and that HCP treatments can downregulate melanin synthesis or transfer-related gene expression by inhibiting Wnt/ β -catenin signaling pathway. HCP may emerge as a safe candidate for treating skin pigmentation disorder in the future.

INTRODUCTION

Skin color mainly depends on the distribution of pigments in the epidermal layer.¹ The variations in skin color among individuals can be attributed to differences in the number, size, composition, and distribution of melanosomes.² Melanosome, a lysosome-like structure in melanocytes, is responsible for the production of melanin.³ Mature melanosomes undergo cytoskeletal transport towards the periphery of melanocytes to facilitate the transfer of melanin content to the surrounding keratinocytes.^{4,5} A protein complex consisting of Rab27A, Mlph/Slac2-a and myosin Va is thought to mediate actin-dependent melanosome transportation in melanocytes.⁶ Dysfunction of Rab27A is known to cause a hypopigmentation phenotype in human type 2 Griscelli syndrome patients, indicating that Rab27A is essential in maintaining normal and well-organized pigmentation.⁷ Additionally, the activation of Rac1 is essential for the formation of melanocytic dendrites, facilitating membrane ruffling, and lamellipodia formation.⁸ While several compounds

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have been employed for skin whitening by inhibiting melanin production, there is still a lack of drugs that specifically target melanin transfer. Current whitening compounds, such as hydroquinone and alpha arbutin, have been reported to be carcinogenic and can cause redness or irritation when used in large amounts.⁹ Therefore, there has been an increasing emphasis on the development of safer whitening products.

Pearl extract is rich in minerals and amino acids. Pearl extracts can enhance skin moisture, skin repair, and UV protection without any unwanted side effects. It has been demonstrated that a soluble pearl extract possesses the ability to inhibit melanin synthesis in B16 melanoma cells.¹⁰ *In vitro* studies have also shown that HCP exhibits anti-inflammatory and anti-apoptotic properties.¹¹ HCP is a hydrolyzing derivative of conchiolin protein extracted from pearls. As a significant active component of pearl extract, HCP has been found to be biocompatible, but the specific effects of HCP on melanin synthesis and transport remain unknown.

In this study, we aimed to investigate the effects of HCP on melanocyte morphology and melanosome transport, and to further elucidate the underlying mechanism. We observed that HCP effectively suppressed melanocyte dendrite extension induced by ET-1 and downregulated the expression of genes involved in melanosome transport, including MITF, Rab27A, and Rac1, suggesting a novel role for HCP in regulating melanosome transport. Additionally, HCP was found to negatively regulate the activity of the Wnt/ β -catenin signaling pathway, leading to the decrease of pigmentation in zebrafish. Therefore, it seems that HCP can inhibit melanin transfer by suppressing the activity of Wnt/ β -Catenin signaling pathway in melanocytes.

MATERIALS AND METHODS

REAGENTS

HCP was obtained from OSM Biology Co., Ltd. (Zhejiang, China). ET-1 was purchased from MedChemExpress Co., Ltd. (Shanghai, China).

CELL CULTURE

PIG1 and MelanA cells were purchased from American Type Culture Collection (ATTC, Virginia, USA) and cultured at 37°C with 5% CO₂ atmosphere in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin.

CELL VIABILITY ASSAY

PIG1 and MelanA cells were cultured in 96-well plates at a density of 1.5×10^5 /ml. Cells were treated with HCP and ET-1 at various concentrations for 24 hours. For PIG1 cells, we selected HCP treatment concentrations of 0, 0.01, 0.1, 5, 10, 20, 50, 80, and 100 µg/ml. For MelanA cells, we selected HCP treatment concentrations of 0, 0.1, 1, 2, 4, 8, 10, 20, 40, 80 µg/ml. After a brief wash with medium, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (0.5 mg/ml in DMEM) was used for the quantification of metabolically active cells, and samples were measured photometrically at 490 nm.

FLOW CYTOMETRIC ANALYSIS

PIG1 and MelanA cells (1×10^6) were trypsinized and washed in ice-cold phosphate buffered saline (PBS). Cells were then fixed with 70% ethanol for 12 hours at 4°C. After washing, cells were resuspended and incubated at 37°C for 30 minutes in 0.5 ml of PBS containing 10 mg/ml propidium iodine (Sigma) and 5 mg/ml RNase A (Sigma). Proportions of cells in either G1 or S phase of cell cycle were analyzed by flow cytometry using a BD FACSCalibur™ (Becton-Dickinson, New Jersey, USA).

IMMUNOFLUORESCENCE ANALYSIS

PIG1 and MelanA cells were fixed with 4% paraformaldehyde for 10 minutes, washed with PBS 2 times, and permeabilized with 0.1% Triton X-100 for 10 minutes. After blocking with 0.2% BSA for 30 minutes, cells were incubated with the prepared FITC-labeled phalloidin working solution or antibody of anti-β-Catenin at room temperature, for 30 minutes in the dark. Fluorescent images were obtained by an Olympus fluorescence microscope (Olympus Corporation, Tokyo, Japan) and the number of melanocyte dendrites was counted.

RNA ISOLATION AND REAL-TIME QUANTITATIVE PCR (RT-QPCR)

Cell RNAs were isolated using the Invitrogen TRIzol® reagent (Thermo Fisher Scientific, Massachusetts, USA). cDNAs were synthesized by using the M-MLV reverse transcriptase kit (Promega Corporation, Wisconsin, USA) and used as the templates for quantitative polymerase chain reaction (qPCR) (MxPro QPCR Software, Agilent Technologies, USA) according to manufacturer instructions. The primers were as followed: *homo glyceraldehyde 3-phosphate dehydrogenase (GAPDH)*: forward, 5'-ACCCAGAAGACTGTGGATGG-3', reverse, 5'-TTCAGCTCAGGGATGACCTT-3'; *homo Rac1*: forward, 5'-GAGACGGAGCTGTTGGTAAAA-3', reverse, 5'-ATAGGCCAGATTCACTGGTT-3'; *homo Rab27a*: forward, 5'-ACAACAGTGGGCATTGATTTC-3', reverse, 5'-AAGCTACGAAACCTCTCCTGC-3'; *mus GAPDH*: forward, 5'-ACCCAGAAGACTGTGGATGG-3', reverse, 5'-TTCAGCTCAGGGATGACCTT-3'; *mus Rac1*: forward, 5'-TCCGCAAACAGATGTGTTCTTA-3', Reverse, 5'-CGCACCTCAGGATACCACTTT-3'; *mus Rab27a*: forward, 5'-TCGGATGGAGATTACGATTACCT-3', reverse, 5'-TTTTCCTGAAATCAATGCCCA-3'. qRT-PCR was performed to detect the expression of related genes according to the following conditions: 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds (40 cycles).

WESTERN BLOT ANALYSIS

Cells were seeded into a 6-well plate at a density of 2×10^5 /ml. 20 nM ET-1 was added to treat cells for 24 hours. Subsequently, cells were treated with different concentrations of HCP. After 24 hours, radio-immunoprecipitation was used for total protein extraction. The extracted proteins were then separated by 10% SDS-polyacrylamide gel and transferred to nitrocellulose filter membranes. After blocking with 5% BSA for 1 hour, membranes were

incubated with the primary antibodies of anti-Rab27A (Abcam, Cambridge, UK), anti-Rac1 (Abcam, Cambridge, UK), anti-MITF (Cell Signaling Technology, Massachusetts, USA), anti- β -Catenin (Cell Signaling Technology, Massachusetts, USA), anti-phospho-GSK-3 β (Cell Signaling Technology, Massachusetts, USA) and anti-tyrosinase (Abcam, Cambridge, UK) at 4°C overnight and then were incubated with the fluorescently labeled secondary antibody for 2 hours at room temperature. The protein bands were visualized by the fluorescent Western blot imaging systems (LI-COR Biosciences, Nebraska, USA).

MEASUREMENT FOR MELANIN SECRETION AND TYR ACTIVITY ASSAY

MelanA cells were maintained in phenol red-free DMEM (Gibco, Massachusetts, USA) that contains 104 mg/L L-Tyrosine disodium salt dehydrate, pretreated with 20 nM ET-1 for 24 hours and then treated with HCP of 1 μ g/ml and 10 μ g/ml for 24 hours. Phenol red-free DMEM without HCP served as a control. Extracellular melanin in the medium secreted by MelanA cells was measured at 490 nm.

MelanA cells were pretreated with 20 nM ET-1 for 24 hours, then treated with different concentrations of HCP for 24 hours. Cells were lysed with 1% Triton X-100 at -80°C for 30 min in 24-well plates. Cell extracts were centrifuged at 12,000 rpm for 10 minutes at 4°C, and the supernatants were used for TYR activity assay, where 100 μ L of 4 mM L-Dopa and the test supernatants were incubated in a 96-well plate for 1 hour at 37°C. Colorimetric measurement was performed at 490 nm.

STATISTICAL ANALYSIS

Statistical analysis of results was performed using one-way analysis of variance (ANOVA) with Tukey's correction for multiple comparisons. All data were analyzed using GraphPad Prism software (GraphPad, California, USA). Values were given as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ were considered significant.

RESULTS

HCP EXHIBITS LOW TOXICITY TO PIG1 AND MELANA CELLS

To investigate the effects of HCP on melanin transfer in PIG1 and MelanA cells, we first confirmed the safety of HCP on these two types of cells using MTS assay. As shown in Figure 1A, no marked cytotoxic effects of HCP were observed even at the concentration of 10 μ g/ml in PIG1 and MelanA cells. In addition, we performed flow cytometry to further detect the effects of HCP on cell cycle. As expected, HCP treatments at the concentrations of 1 μ g/ml and 10 μ g/ml did not alter cell cycle progression of MelanA and PIG1 cells (Figure 1B, Table I). These results together indicate that HCP was safe for MelanA and PIG1 cells.

Given that soluble pearl extract has been shown to lighten the skin by antagonizing endothelin, it is reasonable to hypothesize that HCP, as a crucial active component of pearl extract, may exert its effects by inhibiting endothelin. Thus, we also detected the safe concentration of ET-1 in MelanA and PIG1 cells. As shown in Figure 1C, 0–60 nM ET-1

Table I
The Percentage of Cells in Each Phase of the Cell Cycle in Melana and PIG1 Cells With Different Treatments.

MelanA					
Sample	CTRL	ET-1	ET-1+HCP1	ET-1+HCP10	HCP10
Freq.G0/G1	42.4%	39.2%	40.5%	40.3%	39.9%
Freq.S	35.9%	37.5%	36.9%	36.9%	40.3%
Freq.G2/M	15.9%	17.9%	17.9%	17.1%	13.3%

PIG1					
Sample	CTRL	ET-1	ET-1+HCP1	ET-1+HCP10	HCP10
Freq.G0/G1	36.9%	36.7%	35.1%	32.0%	32.1%
Freq.S	35.0%	30.9%	33.9%	34.7%	34.2%
Freq.G2/M	20.8%	24.3%	22.5%	22.8%	22.9%

caused no marked cytotoxicity in PIG1 cells, while 0-90 nM ET-1 had no toxic effects on MelanA cells. Therefore, 20 nM ET-1 was selected for further experiments.

HCP INHIBITS MELANIN TRANSFER OF PIG1 AND MELANA CELLS

It is reported that the formation and growth of melanocyte dendrites are the prerequisites for the transfer of melanosomes. To assess the potential impact of HCP on melanin transfer in melanocytes, we examined alterations in melanocyte morphology following HCP treatments. As shown in Figure 2A, 20 nM ET-1 significantly increased the number of cell dendrites when compared to the control. However, when 1 μ g/ml or 10 μ g/ml HCP were applied, the number of dendrites significantly decreased. After treating the cells with ET-1, there was a significant increase in the proportion of cells with dendrites equal to or greater than three. However, after 24 hours of HCP treatment, this ratio significantly decreased in a concentration-dependent manner (Figure 2B). A similar trend was also observed in MelanA cells (Figures 2C and 2D). These results demonstrated that HCP can greatly inhibit ET-1-induced increase in the number and length of dendrites in MelanA and PIG1 cells, indicating its role in inhibiting melanin transfer. To further confirm the impact of HCP on melanin transfer, we assessed melanin secretion from cultured MelanA cells into a phenol red-free medium. As expected, 20 nM ET-1 markedly increased melanin content in the medium, while HCP treatment effectively reversed the ET-1-induced increase of melanin in the medium and fully returned it to the normal level (Figures 2E and 2F). Together, all the data show that HCP possesses a new role of inhibiting melanin transfer in melanocytes.

HCP INHIBITS MELANOSOME TRANSPORT-RELATED GENE EXPRESSION

Rac1 is one of the key factors in the transport of melanosomes from melanocytes.¹² Rab27a has been found to be anchored to melanosomes and promotes the movement of melanosomes along actin filaments.¹³ Thus, we performed Western blot experiments to detect whether HCP influenced the expression of these two key factors involving in melanosome transport. As shown in Figures 3A to 3C, ET-1 elevated the mRNA and protein levels of Rac1

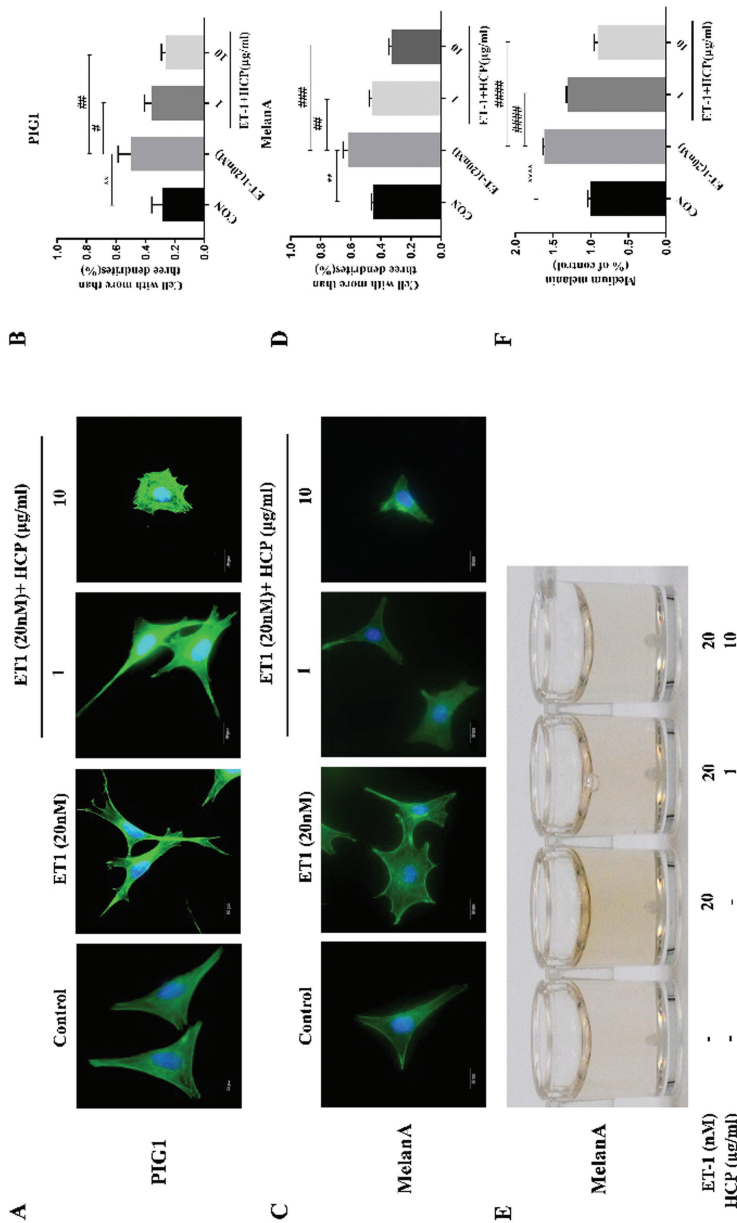


Figure 2. HCR inhibits dendrite formation of PIG1 and MelanA cells. Representative photoes of cell morphology of PIG1 (A) and MelanA (C) cells. PIG1 cells were pretreated with 20 nM ET-1 for 24h, then treated with different concentrations of HCP for 24h. Cytoskeleton was visualized by cell immunofluorescent staining for F-actin. Scale bars = 20 μm. The number of dendrites were counted in PIG1 (B) and MelanA (D) cells. (E) Melanin secretion to the medium in MelanA cells treated with 20 nM ET-1 in the presence or absence of HCP. (F) Quantitative analysis of melanin content in the medium. Data were expressed mean ± SD from 3 independent experiments (n = 3). *P < 0.05, **P < 0.01, ET-1 vs control; #P < 0.05, ##P < 0.01, HCP versus ET-1.

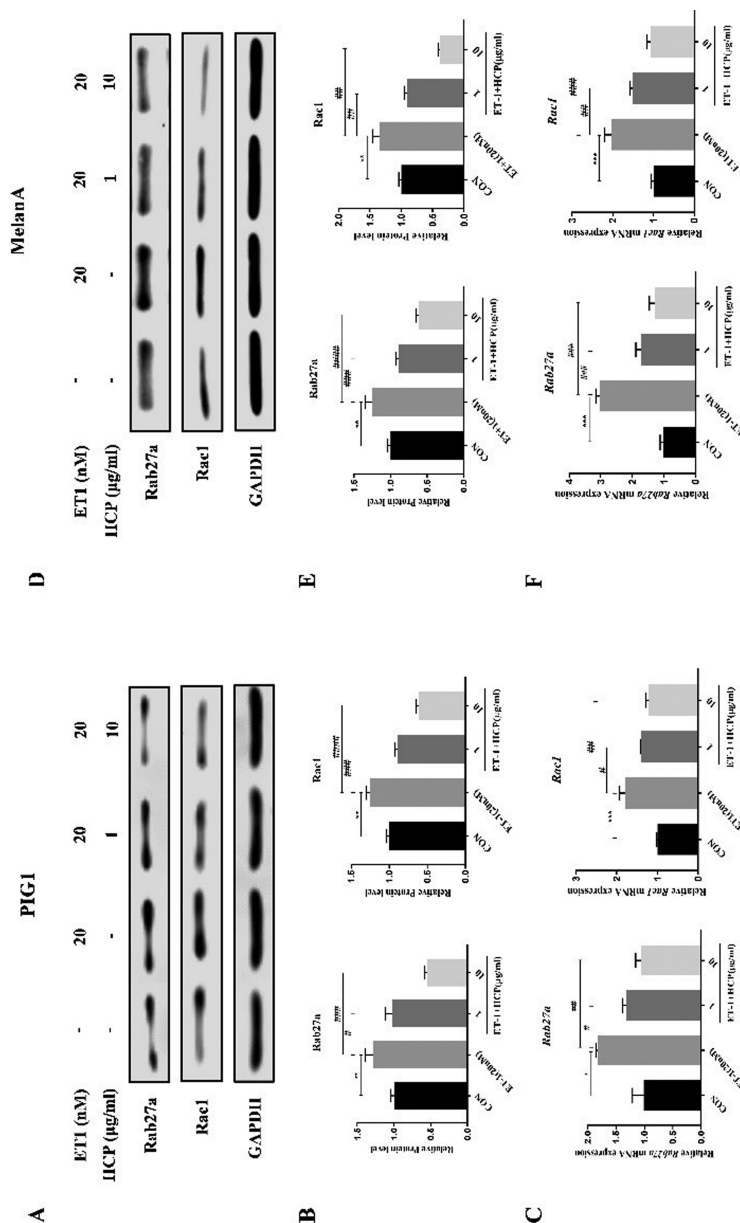


Figure 3. HCR inhibits melanin transfer-related gene expression. PI31 (A) and MelanA (D) cells were pretreated with 20 nM ET-1 for 24h, then treated with different concentrations of HCP for 24h. Rab27a and Rac1 levels were determined by Western blot analysis. Relative expression levels of Rab27a and Rac1, normalized to the expression of GAPDH, were quantified by Image J software in PI31 (B) and MelanA (E) cells. The expression of Rac1 mRNA in PI31 (C) and MelanA (F) cells treated with ET-1 or HCP were quantified by using real-time PCR analysis. Data were expressed mean $\pm$ SD from 3 independent experiments ($n = 3$). * $p < 0.05$, *** $p < 0.001$ ET-1 vs control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, HCP versus ET-1.

and Rab27a in PIG1 cells, while HCP treatments significantly inhibited the expression of Rac1 and Rab27a induced by ET-1 in a dose-dependent manner. Consistently, we also demonstrated that HCP could inhibit the expression of Rac1 and Rab27a in MelanA cells both at the mRNA and protein levels (Figures 3D–F). These findings suggest that HCP may inhibit the transport of melanosomes in MelanA and PIG1 cells by downregulating gene expression of Rac1 and Rab27a.

HCP INHIBITED ET-1-INDUCED ACTIVATION OF WNT/ β -CATENIN SIGNAL PATHWAY

It is reported that MITF not only controls the expression of factors related to melanin synthesis but also plays a role in regulating the expression of Rab27A.¹⁴ Therefore, we investigated the impact of HCP on the expression of MITF in melanocytes. As shown in Figures 4A and 5A, 20 nM ET-1 notably increased the expression of MITF in PIG1 cells, while HCP effectively reversed the increase of MITF expression induced by ET-1. Similar results were also observed in MelanA cells (Figures 4B and 5B). TYR, the key enzyme involved in melanin synthesis, is recognized as a target gene of MITF. This is attributed to the presence of an E-box motif within the TYR promoter, which facilitates the binding of the transcription factor MITF. Consistently, the gene expression and activity of TYR were also inhibited by HCP treatments in MelanA cells (Figure 6A–C).

Considering that the expression of MITF can be regulated by the Wnt/ β -catenin signaling pathway, we further investigated the effects of HCP on this signaling pathway. As shown in Figures 4A and 4B, the level of phosphorylation GSK-3 β and β -catenin in PIG1 cells was significantly increased by ET-1, while 1 μ g/ml and 10 μ g/ml HCP treatment greatly reversed the ET-1-induced increase of p-GSK-3 β and β -catenin. GSK-3 β can combine with β -catenin to form a complex, keeping β -catenin in an inactive state. As expected, HCP exhibited a concentration-dependent inhibitory effect on the ET-1-induced increase in β -catenin expression. Importantly, the increase of ET-1-induced β -catenin nuclear localization in both PIG1 and MelanA cells was greatly inhibited by HCP (Figures 4C and 4D; Figure 6C). These data indicate that HCP treatments can inhibit the activation of Wnt/ β -catenin signaling pathway induced by ET-1. Therefore, it is inferred that HCP may suppress ET-1-induced dendrite formation in melanocytes by inactivating the Wnt/ β -catenin signaling pathway.

DISCUSSION

The metabolism of melanin is a systematic process involved in melanin production, transport, uptake, and degradation.² Both melanogenesis and melanin transfer contribute to the accumulation of melanin in the skin. A previous study reported that soluble pearl extract was able to inhibit the expression of TYR in B16 melanoma cells, indicating its potential as a skin whitening agent.¹⁰ However, the specific impact of HCP (the most abundant protein in pearl powder) on melanin synthesis and transfer remains unclear. In this study, we find that HCP can reduce melanocyte dendrites and melanin secretion by antagonizing the effects of ET-1. Additionally, HCP has shown the ability to downregulate melanosome transport-related gene expression and inhibit the activity of the Wnt/ β -catenin signal pathway. Our results demonstrate that HCP seems to inhibit melanin transfer by regulating the Wnt/ β -catenin signal pathway. It is important to note that HCP, at a

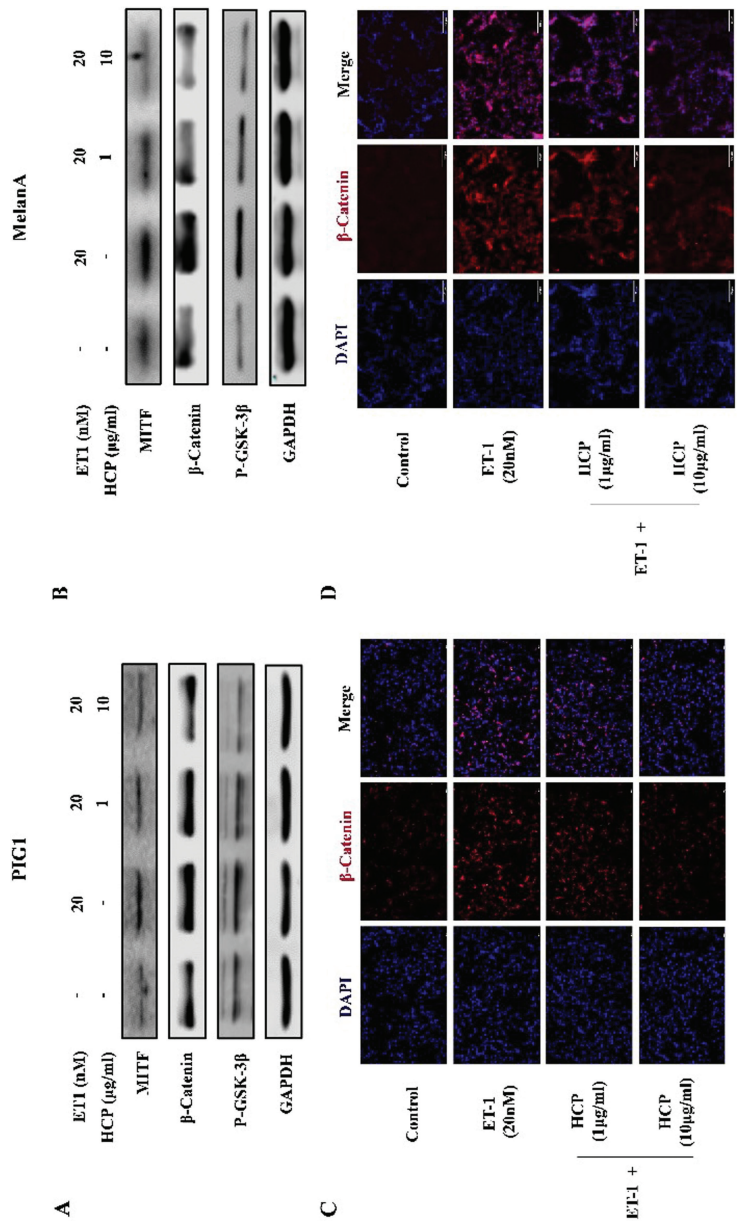


Figure 4. HCP inhibits the activity of Wnt/ β -catenin pathway. PI31(A) and MelanA (B) cells were pretreated with 20 nM ET-1 for 24h, then treated with different concentrations of HCP for 24h. β -Catenin, p-GSK-3 β and MITF protein expression were determined by Western blot analysis. HCP treatment leads to a reduction in the nuclear localization of β -catenin. Immunostainings of β -catenin of PI31 and MelanA cells after 20 nM ET-1 treatments with or without HCP. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) blue. Scale bars = 100 μ m.

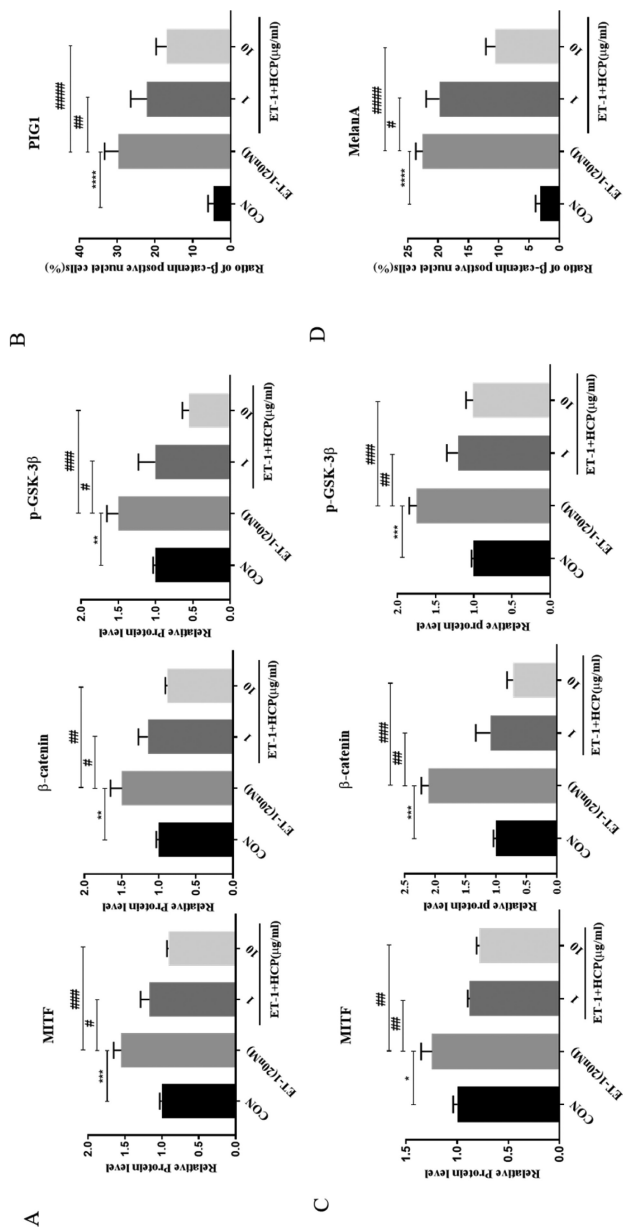


Figure 5. The effects of HCP on Wnt/ β -catenin pathway. Quantification of β -Catenin, p-GSK-3 β and MITF protein levels in PI31 (A) and MelanA (B) cells by Image J software for Figure 4A and 4B. Graph of β -catenin positive nuclei percentage quantification in PI31 (C) and MelanA (D) cells for Figure 4C and 4D. *** $p < 0.001$, ET-1 versus dimethyl sulfoxide (DMSO) control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, HCP versus ET-1.

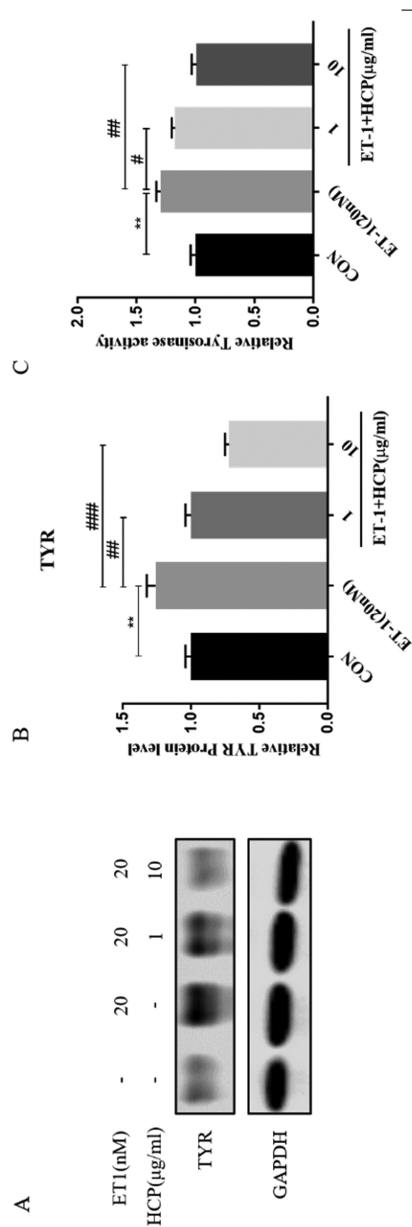


Figure 6. The effects of HCP on TYR expression and activity. (A) MelanA cells were pretreated with 20 nM ET-1 for 24h, then treated with different concentrations of HCP for 24h. TYR expression were determined by Western blot analysis. (B) Quantification of tyrosinase protein expression in MelanA cells by Image J software. (C) The activity of tyrosinase in MelanA cells. Data were expressed mean $\pm$ SD from 3 independent experiments (n = 3). **p < 0.01 ET-1 vs control; # p < 0.05, ## p < 0.01, ### p < 0.01, HCP versus ET-1.

moderate concentration, did not hinder cell progression or induce abnormal cell growth, providing evidence of its safety as a potential skin whitening agent for future clinical applications.

Several studies showed that melanin in melanosomes moves toward the cell membrane along the cytoskeleton, in which Rab27a and Rac1 play important roles.^{12,13} Here, we demonstrated that HCP can significantly inhibit the expression of Rab27a and Rac1 in PIG1 and MelanA cells, indicating the potential of HCP in blocking melanin transfer. The significant reduction in the number of cell dendrites provides further evidence supporting the potential role of HCP in modulating melanosome transfer processes. We also observed that HCP effectively inhibited the increase of ET-1-induced melanin secretion to the medium in MelanA cells. Taken together, all the data indicate that HCP possesses a novel function in inhibiting melanin transfer in melanocytes.

MITF is a critical transcription factor in melanin synthesis, as it plays a central role in regulating the expression of key proteins involved in melanin synthesis, including TYR and its related proteins.¹⁴ In addition, MITF can bind to the promoter of Rab27A and activate its transcription.¹⁵ In this study, we found that HCP significantly inhibited MITF expression in PIG1 and MelanA cells. Based on the evidence of reduced TYR expression and activity in MelanA cells, it can be inferred that HCP potentially exerts a dual role in both inhibiting melanin synthesis and suppressing melanin transfer processes.

MITF is regulated by multiple upstream signaling pathways, among which the Wnt/ β -catenin signaling pathway is known to play a role.¹⁶ Interestingly, we found that the expression of p-GSK-3 β and β -catenin was significantly decreased after HCP treatments. Critically, HCP treatments were able to block the nuclear entrance of β -catenin induced by ET-1 in both PIG1 and MelanA cells. Obviously, HCP may inhibit melanocyte dendrite formation and melanin transfer by inactivating the Wnt/ β -catenin signaling pathway. Several studies have reported that the cAMP-dependent protein kinase (PKA) signaling pathway can also regulate MITF expression and impact Rac1 phosphorylation.^{17,18} The potential relations between HCP and PKA signal pathway will be investigated in the future.

ET-1 is known as a melanogen in UVB-exposed human epidermis.¹¹ In this study, we demonstrated that ET-1 can enhance the formation of melanocyte dendrite, upregulate gene expression of MITF, Rab27a, and Rac1, and enhance the activity of the Wnt/ β -catenin signaling pathway. Previous studies showed ET-1 was able to induce MITF expression in human melanocytes and enhance β -catenin activity in colorectal cancer, which is consistent with our results.¹⁹ Importantly, HCP can fully reverse all the molecular responses induced by ET-1 in PIG1 and MelanA cells. These results indicate that HCP has the potential to act as an effective antagonist of ET-1, thereby inhibiting melanin synthesis and transfer in both PIG1 and MelanA cells.

CONCLUSION

In summary, our study is the first to demonstrate that HCP treatments effectively suppress melanin transfer in melanocytes by antagonizing ET-1. HCP treatments can also downregulate melanin synthesis or transfer-related gene expression by inhibiting the Wnt/ β -catenin signaling pathway, which eventually leads to the suppression of dendrite formation and TYR activity (Figure 7). Our results show that HCP may be a potential

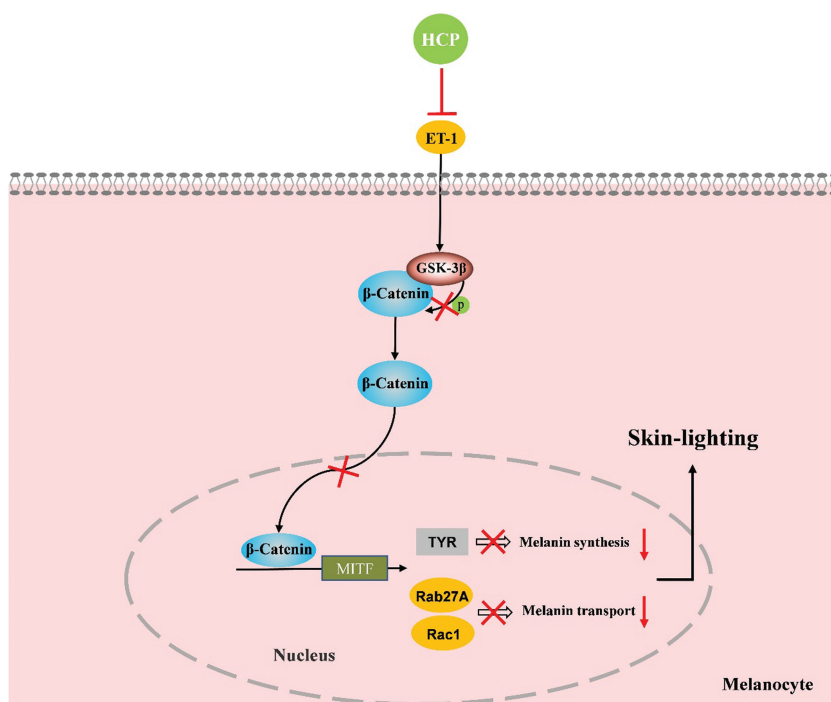


Figure 7. A model depicting the molecular mechanism by which HCP inhibits melanosome transfer in melanocytes through regulating the Wnt signaling pathway.

endothelin antagonist without unwanted side effects. This suggests that HCP may have promising applications as a safer skin whitening agent in the future.

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