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Melanin Uptake Reduces Cell Proliferation of Human Epidermal Keratinocytes

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Abstract

Melanin, synthesized by melanocyte, is transferred to neighboring keratinocyte and finally accumulates in perinuclear site. Except functioning as an internal sunscreen to protect from UV damage, the potential effect of melanin on modulating the bioactivity of keratinocyte has not yet been fully investigated. In this study, we added melanin directly to the culture of human epidermal keratinocytes and the uptake of melanin was found to be dose- and time-dependent as determined by spectrophotometric method. The uptaken melanin accumulated perinuclearly in keratinocytes that is similar to the pattern observed in human solar lentigo tissue by microscopic examination. Pretreatment of keratinocytes with either niacinamide or trypsin inhibitor reduced the uptake of melanin dose-dependently, indicating a PAR-2-dependent pathway involved. Melanin uptake by keratinocytes inhibited cell proliferation as demonstrated both by the decrease of cell number and nuclear Ki-67 expression. Inhibited Ki-67 expression in melanin-containing keratinocyte was also found in human lentigo tissue. The cell cycle arrested at G1 phase in melanin-uptaken keratinocytes was confirmed by flow cytometric method. The protein expressions of cyclin-dependent kinase 1 (CDK1), CDK2, cyclin E, cyclin A and cyclin B were significantly reduced by melanin treatment. Microarray analysis, RT/real-time PCR and western blot demonstrated the inhibited expression of DKK1, a protein known to reduce skin pigmentation, in melanin-uptaken keratinocytes. Together, the direct incubation of keratinocyte with melanin might serve as a useful model to study the potential mechanisms involved in melanin uptake and pigmentation process.

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Keywords

Melanin, Epidermal Keratinocyte, Proliferation, Ki67, Cell Cycle, DKK1

1. Introduction

Melanocytes produce specific organelles, termed melanosomes, in which melanin pigment is synthesized and deposited. Melanin is produced within melanosomes which later migrate into the melanocyte's dendrite tips using myosinV and dynein [1]. It is well known that tyrosinase is a rate limiting step in melanin production but melanosome transfer is also a decisive step responsible for melanogenesis [2] [3]. Therefore, instead of tyrosinase inhibitors that are mostly applied, a few hypopigmenting agents are now intended to inhibit the transfer of melanosomes [4]-[6].

Different mechanisms of melanosome transfer have been proposed including direct infusion of melanosomes into keratinocytes through nanotubular filopodia [7], uptake of released melanosomes by keratinocytes via phagocytosis [8], and partial cytophagocytosis of melanocyte dendrite tips containing melanosomes by keratinocytes [9]. A differernt mechanism for the transfer of melanosomes was reported in which pigment globules containing multiple melanosomes are released into the extracellular space from melanocytes and are then ingested by keratinocytes [10]. Recently, keratinocytes are found to predominantly induce a Rab11b-mediated exocytosis through remodeling of the melanosome membrane before subsequent endocytosis of melanin [11]. No matter which mechanism is acting, the result is the cellular uptake of melanin and finally forms perinuclear melanin caps [12].

Melanin, a natural pigment produced by stimulated melanocytes in the basal layer of skin epidermis, converts more than 99.9% of the absorbed UV light to heat and functions as an excellent photoprotectant that prevents cells from the DNA damage by UV [13]-[15]. Except functioning as an internal sunscreen, the potential effect of melanin on modulating the bioactivity of epidermal keratinocytes has not yet been fully investigated.

In this study, we attempted to investigate the biological effect of melanin in epidermal keratinocytes. Epidermal keratinocytes were incubated with melanin and the direct uptake of melanin was examined and compared with that in human lentigo tissues. Cell proliferation and the expressions of cell cycle-dependent genes were studied. Microarray analysis was carried out to further understand the effect of melanin uptake on the biological function of keratinocytes.

2. Materials and Methods

2.1. Cell Culture

Human epidermal keratinocytes were isolated from skin by enzymatic digestion method as described previously [16]. Skin samples were obtained from donors with informed consents, under the approval of the Institutional Review Board of Chang Gung Memorial Hospital in Tao-Yuan, Taiwan. Briefly, skin was rinsed in 10% iodine for 30 sec and cleaned with 1 × PBS, then incubated in dispase (type II, 2.5 mg/ml in 1 × PBS) for 2 h at 37˚C to dissociate the epidermis from dermis. The epidermal layer was peeled off carefully and cut into small pieces and incubated in 0.25% trypsin for 30 min at 37˚C to release single cell. Cells were centrifuged and the cell pellet was resuspended in keratinocyte serum-free medium (KSFM, GIBCO®) containing EGF and bovine pituitary extract and maintained at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. Keratinocytes between passages 3 and 5 were used for all the experiments. In the present study, epidermal keratinocytes at 70% - 80% confluence were treated with melanin (0 - 100 μg/ml) for 24 h or indicated time and used for the analysis. All experiments were performed in triplicate.

2.2. Measurement of Melanin Uptake

The content of melanin uptake in keratinocytes was determined by spectrophotometric method. Cells were washed thoroughly by 1 × PBS, homogenized with 1N NaOH and heated at 95˚C for 30 min to dissolve intracellular melanin. The melanin content was determined by measuring the absorbance at 495 nm using a spectrophotometer.

2.3. Examination of Melanin Uptake in Cultured Keratinocytes and Solar Lentigo Tissue

Keratinocytes cultured on coverslips were fixed in 1 × PBS containing 4% paraformaldehyde for 15 min and stained with modified Wright-Giemsa stain (Sigma) for the microscopic examination of melanin uptake in cells. HE-stained tissue sections of solar lentigo was obtained from the tissue bank in Chang Gung Memorial Hospital (Tao-Yuan, Taiwan) and pathology was diagnosed and identified by Dr. Wen-Rou Wong who is a skin specialist. Solar lentigo is a small pigmented spot with a clearly defined edge on the skin associated with aging and exposure to ultraviolet radiation from the sun. Histologically, the hallmark of solar lentigo is an increase in basal melanin.

2.4. MTT Assay

Epidermal keratinocytes grown in 24-well culture plate with or without melanin treatment for 24 h were washed once with 1 × PBS, followed by addition of 0.5 ml KSFM containing 0.05 mg/ml 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT). After incubation at 37˚C for 1 h, the medium was removed and formazan crystals in the cells were dissolved in 1 ml DMSO for OD reading at 570 nm using a spectrophotometer.

2.5. Immunofluorescent Staining

Keratinocytes cultured on coverslips were fixed in 4% paraformaldehyde in 1 × PBS for 15 min and then in methanol at −20˚C for 10 min. Cells were blocked in 1 × PBS containing 1% BSA and 1% goat serum for 30 min. Rabbit antibody against Ki67 (Lab Vision) was added to blocking solution in a ratio of 1:50 and incubated at 4˚C overnight. After washing with 1 × PBS, FITC-conjugated goat anti-rabbit IgG (BioFX) was added (1:2000 in 1 × PBS) for 30 min. After washing with 1 × PBS, cell nuclei were counter-stained by propidium iodide for 10 min at 37˚C. Coverslips were mounted with fluorescent specific medium for the examination under fluorescent microscope.

2.6. Immunohistochemistry

Rehydrated tissue section was pretreated in 10 mM citrate buffer, pH 6.0, at 95˚C for 20 min followed by cooling at room temperature for 20 min. Tissue section was blocked in 1 × PBS containing 1% BSA and 1% goat serum for 30 min. Rabbit antibody against Ki67 (Lab Vision) was added to blocking solution in a ratio of 1:50 and incubated at 4˚C overnight. After washing with 1 × PBS, section was incubated with biotinylated goat anti-rabbit IgG (Thermo Scientific) for 15 min. After washing with 1 × PBS, section was incubated with streptavidin-conjugated peroxidase (Thermo Scientific) for 10 min. In order to differentiate from the brown color of melanin in lentigo tissue, positive signals were developed by reacting peroxidase with ImmPACT™ SG substrate (VECTOR) for 15 min and Ki67 expression was developed into blue color. Cell nuclei were counterstained by nuclear fast red (VECTOR) for 10 min and mounted with VectaMount™ AQ mounting medium (VECTOR).

2.7. Flow Cytometry

Keratinocytes were harvested by trypsinization. After washing with 1 × PBS and centrifugation, the cell pellet was resuspended in 1 ml ice-cold 70% ethanol and incubated overnight on a rocker at 4˚C. Cells were collected, washed and incubated in a solution of 0.5% Triton X-100 and 0.05% RNase at 37˚C for 1 h. Cell nuclei were then stained by propidium iodide (PI) solution (50 mg/ml PI in 1 × PBS) and incubated at 4˚C for 20 min. Cell cycle distribution was quantified with a FACS Calibur system (Becton-Dickinson). Percentages of cells in the G_0/G_1, S, and G_2/M phases of the cell cycle were determined and analyzed using CellQuest Pro software (Becton Dickinson).

2.8. RNA Isolation and RT/Real-Time PCR

Total RNA was extracted from keratinocytes by acid guanidinium thiocyanate–phenol–chloroform extraction method, and complementary (c) DNA was synthesized using 1 μg total RNA in a 20 μl RT reaction mix containing 0.5 μg/μl of random primers, 0.1 mM dNTP, 0.1 M DTT and 5× first strand buffer. Real-time PCR was performed using an SYBR Green I technology and MxPro- Mx3000P QPCR machine (Stratagene), and a master

mix was prepared with Smart Quant Green Master Mix with dUTP & ROX Kit (Protech). Relative gene expressions between experimental groups were determined using MxPro software (Stratagene) and GAPDH was used as an internal control. All real-time PCRs were performed in triplicate, and changes in gene expressions were reported as multiples of increases relative to the controls. The following primers were used: GAPDH: 5'-GAGGGGCCATCCACAGTCTT-3' (forward) and 5'-TTCATTGACCTCAACTACAT-3' (reverse). DKK1: 5'-CGTTGTTACTGTGGAGAA-3' (forward) and 5'-GTGTGAAGCCTAGAAGAAT-3' (reverse).

2.9. Western Blot Analysis

Epidermal keratinocytes were rinsed with cold 1 × PBS, scraped and solubilized in lysis buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin), followed by sonication and centrifugation at 12,000 g for 20 min at 4˚C. The protein concentration in the supernatant was determined by Bradford protein assay kit (Bio-Rad). Cell lysates containing equal amounts of protein were separated by 10% SDS-PAGE, and transferred onto PVDF membrane (Millipore). The membrane was incubated in blocking solution (1% BSA, 1% goat serum in 1 × PBS) for 1 h, followed by incubation with primary antibody diluted in blocking solution. After washing, the membrane was incubated in 1 × PBS containing secondary antibody conjugated with horseradish peroxidase for 1 h. The membrane was washed and the positive signals were developed with enhanced chemiluminescence reagent (Amershan Pharmacia Biotech). The following primary antibodies were used: anti-CDK1 antibody (MS-110-P1, Thermo), anti-CDK2 antibody (MS-459-P0, Thermo), anti-cyclin A antibody (ab38, Abcam), anti-cyclin B antibody (MS-338, Thermo), anti-cyclin E antibody (14-6714, eBioscience), anti-DKK1 (AF1096, R&D systems) and anti-tubulin-α antibody (MS-581-P, Thermo). Tubulin was used as the sample loading control.

3. Results

3.1. Direct Uptake of Melanin in Human Epidermal Keratinocytes

In order to study the direct effect of melanin on the cellular function of epidermal kerationcytes, we incubated keratinocytes with melanin in culture medium for 24 h and the uptake of melanin was measured by spectrophotometric method. The uptake of melanin by keratinocytes occurred soon after the addition of melanin for 1 h and the extent of uptake significantly increased in a dose- and time-dependent manner (**Figure 1(a)** and **Figure 1(b)**, respectively). The uptaken melanin was revealed to be accumulated surrounding the nucleus (**Figure 1(c)**) which was similarly observed in human lentigo tissue (**Figure 1(d)**) as indicated by yellow arrows.

3.2. Niacinamide and Trypsin Inhibitor Suppressed Melanin Uptake in Epidermal Keratinocytes

To understand the possible mechanism for epidermal keratinocytes to directly uptake melanin, we tested two well-known inhibitors for the transfer and uptake of melanosome into epidermal keratinocytes on this simplified cell model. Epidermal keratinocytes were pretreated with niacinamide or trypsin inhibitors for 3 h and then 50 μg/ml melanin was added for 24 h. The amount of melanin uptaken by epidermal keratinocytes was demonstrated to be dose-dependently suppressed by niacinamide or trypsin inhibitor (**Figure 2(a)** and **Figure 2(b)**, respectively).

3.3. Melanin Inhibited Cell Proliferation and Ki-67 Expression in Epidermal Keratinocytes

The uptake of melanin appeared to reduce the cell density of epidermal kertinocytes from microscopic observation. Epidermal keratinocytes with different concentration of melanin treatment for 24 h were analyzed by MTT assay. Results in **Figure 3(a)** demonstrated the dose-dependent effect of melanin on decreasing the cell number of epidermal keratinocytes. To further prove the inhibitory effect of melanin on the cell proliferation of epidermal keratinocytes, the expression of Ki-67, a cell proliferating marker, was fluorescently stained in epidermal keratinocytes. As clearly shown in **Figure 3(b)**, the number of cells with positively stained Ki-67 expression (green fluorescent signal) in the nucleus was significantly decreased after melanin treatment. The dose-dependent effect of melanin on suppressing the Ki-67 expression in keratinocytes was calculated and confirmed in

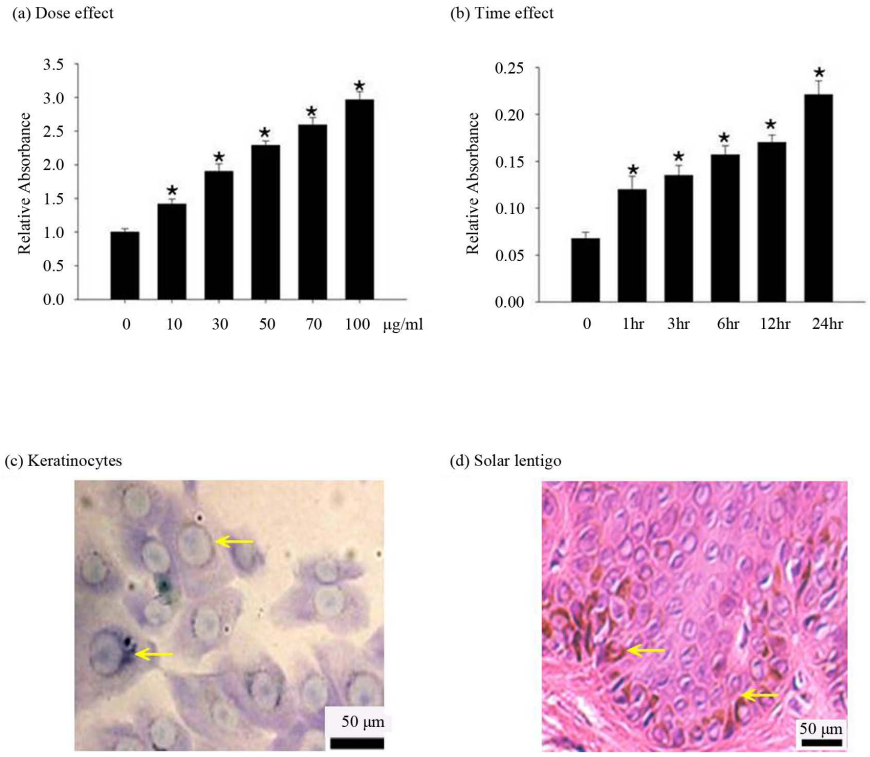

Figure 1. Melanin uptake in human epidermal keratinocytes was dose- and time-dependent. (a) Melanin at the concentration from 0 to 100 μg/ml as indicated was added to 70% - 80% confluent human epidermal keratinocytes for 24 h and the melanin uptake was measured by spectrophotometric method. (b) Melanin at the concentration of 100 μg/ml was added to 70% - 80% confluent human epidermal keratinocytes and the melanin uptake was measured by spectrophotometric method at 0, 1, 3, 6, 12 and 24 h. The perinuclear accumulation of melanin was observed in (c) cultured human epidermal keratoinocytes and in (d) human lentigo tissue.

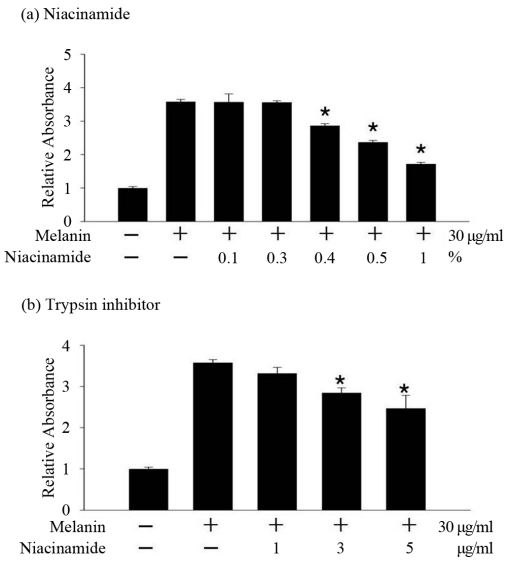

Figure 2. Niacinamide and trypsin inhibitor reduced the melanin uptake in epidermal keratinocytes. Human epidermal keratinocytes at 70% - 80% confluency were pretreated with (a) niacinamide (0% - 0.5% as indicated) and (b) trypsin inhibitor (0 - 10 μg/ml as indicated) for 3 h and then melanin at the concentration of 50 μg/ml was added for 24 h. Melanin uptake was measured by spectrophotometric method.

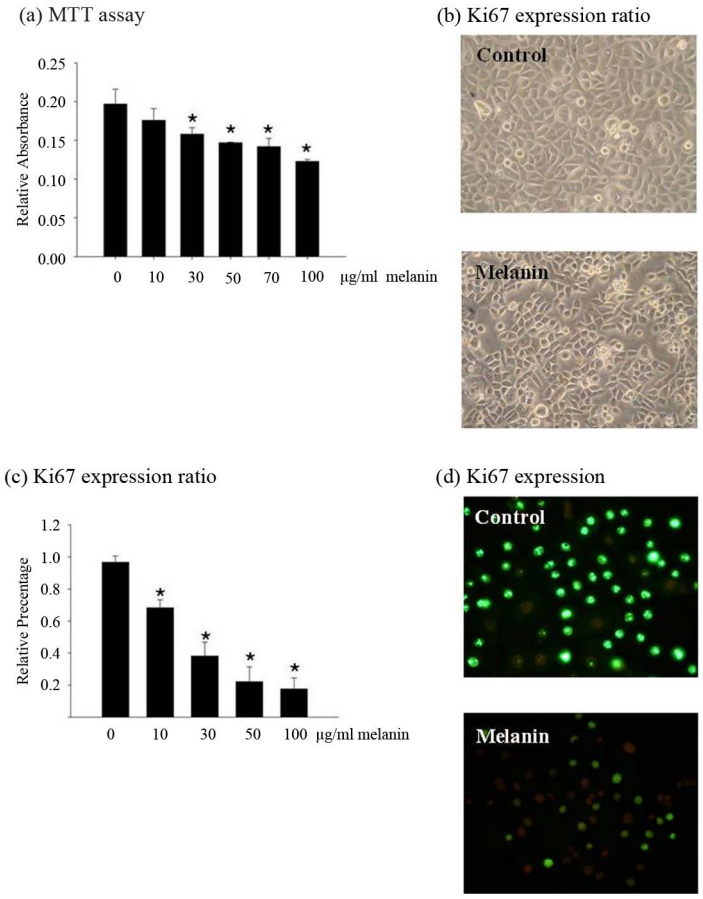

Figure 3. Melanin inhibited the proliferation of human epidermal keratinocytes. (a) Melanin at the concentration from 0 to 100 μg/ml as indicated was added to 70% - 80% confluent human epidermal keratinocytes for 24 h and MTT assay was performed. (b) Representative photo for the fluoresecently labeled Ki-67 expression in epidermal keratoinocytes without (upper panel) or with (lower panel) 50 μg/ml melanin treatment for 24 h. (c) The dose-dependent effect of melanin on the number of Ki-67 positive epidermal keratonicytes was quantified.

Figure 3(c). Similar correlation was also found in human solar lentigo tissue as shown in **Figure 4**. Yellow arrow pointed out the cell with perinuclear accumulation of melanin (brown color) in which Ki67 expression was observed to be reduced. On the other hand, cell with positive Ki67 signal (blue color) as pointed out by red arrow was found to contain less melanin accumulation in the cells.

3.4. Melanin Arrested the Cell Cycle Progression of Epidermal Keratinocytes

The inhibitory effect of melanin on the proliferation of epidermal keratinocytes was further confirmed by the cell cycle distribution analysis using flow cytometry. Results in **Figure 5(a)** clearly revealed that the melanin treatment resulted in the increase of G1phase-cells from 41.84% to 57.57% and the decrease of S phase-cells from 45.52% to 30.09%. Clearly, the cell cycle progression of epidermal keratinocytes was delayed and partially arrested at G1 phase by melanin. The expressions of cell cycle-dependent genes including CDK1, CDK2, cyclin E, cyclin A and cyclin B were dose-dependently suppressed in epidermal keratinocytes by the treatment with melanin for 24 h (**Figure 5(b)**).

3.5. Microarray Study Revealed the Down-Regulation of DKK1 Gene Expression in Human Epidermal Kerationocytes by Melanin

To further explore the potential effect of melanin on the biological functions of epidermal keratinocytes, we

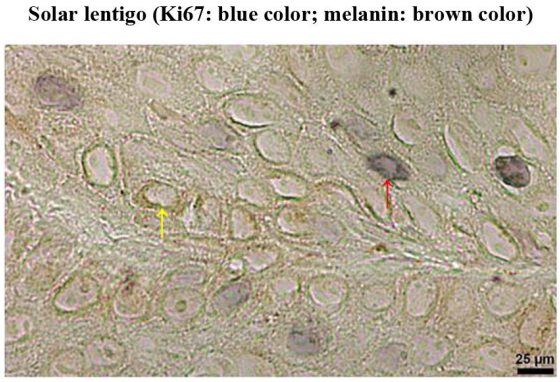

Figure 4. Melanin inhibited the in vivo Ki-67 expression in keratinocytes. The Ki-67 expression in human lentigo tissue was revealed by immunostaining the protein and developed into blue color. Yellow arrow indicated the cell with melanin uptake and red arrow indicated the cell without melanin uptake. Representative photo was shown from five different lentigo tissues with similar results.

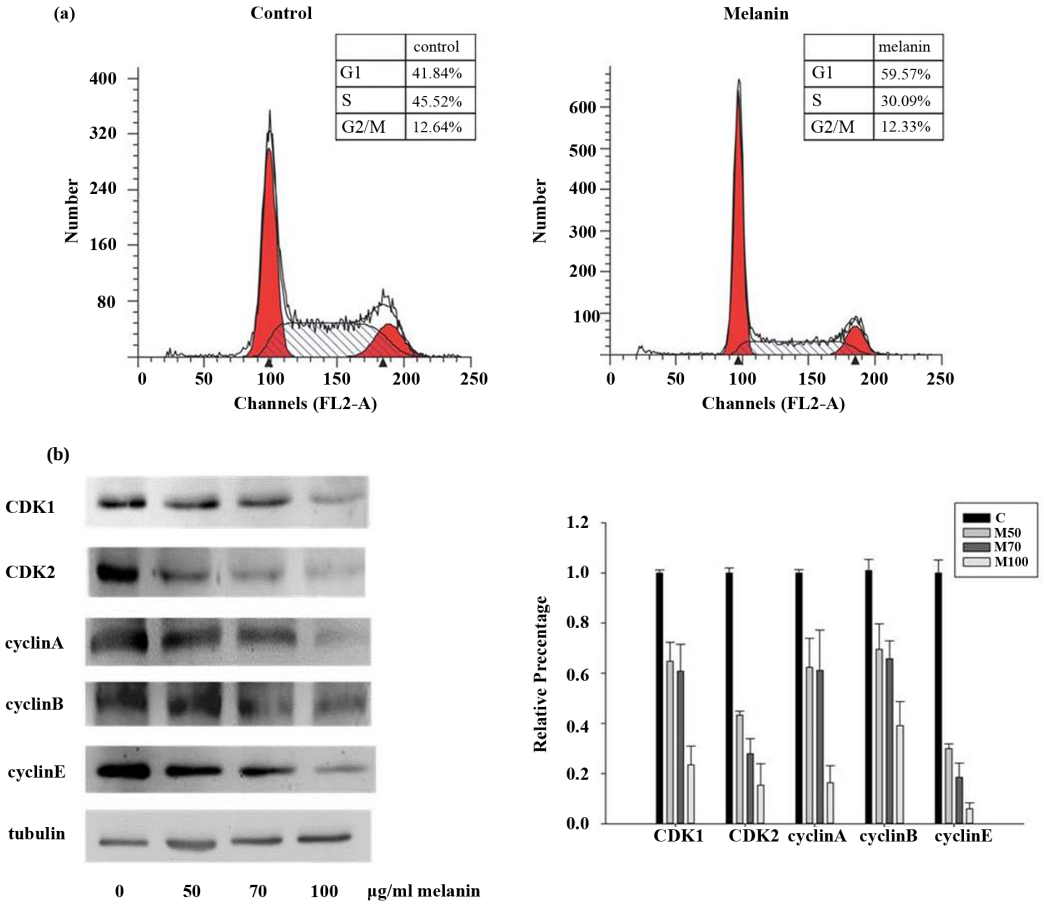

Figure 5. Melanin inhibited cell cycle progression and the gene expressions of cell cycle-dependent genes. (a) Epidermal keratinocytes were treated with 50 μg/ml melanin for 24 h and processed for the analysis of cell cycle distribution by flow cytometry. (b) Protein lysates were prepared from cells treated with different concentrations of melanin and western blot analysis was performed.

isolated RNA isolated from this simplified cell model and microarray analysis was performed by Phalanx Biotech Group (Hsinchu, Taiwan, R.O.C.) using The Human Whole Genome One Array® v6 containing 32,679 DNA oligonucleotide probes to reveal the gene expression profile changed by melanin. Results shown in **Figure 6(a)**

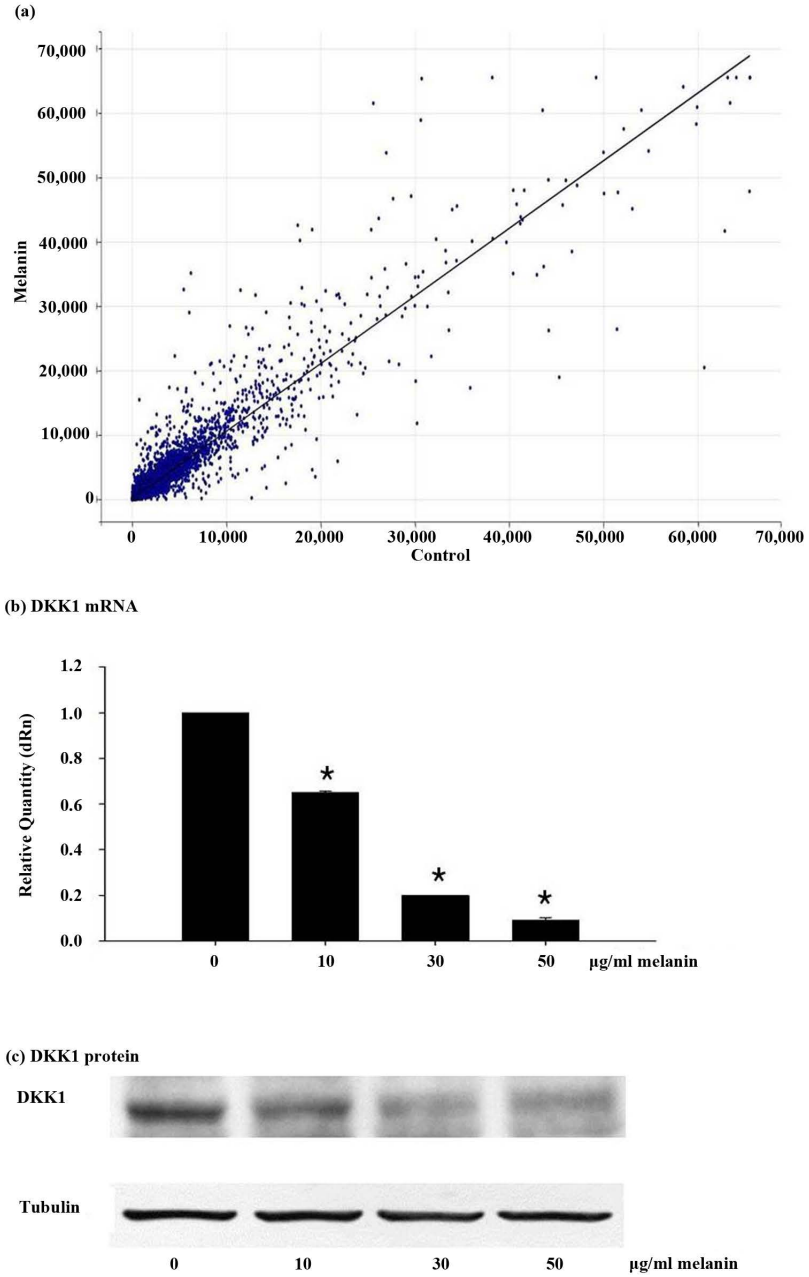

Figure 6. Microarray analysis revealed the inhibition of DKK1 gene expression in keratinocytes by melanin. (a) Full microarray data of expressed gene probes plotted as melanin-treated cells mean signal vs. control mean signal. Melanin at the concentration from 0 to 50 μg/ml as indicated was added to 70% - 80% confluent human epidermal keratinocytes for 24 h and the mRNA level was measured by (b) RT/real-time PCR and (c) western blot analysis, respectively.

revealed 800 genes with more than two-fold changes in melanin-treated keratinocytes. Among them, DKK1 was found to be remarkably suppressed in epidermal keratinocytes by melanin. In **Figure 6(b)** and **Figure 6(c)**, the mRNA and protein expression levels of DKK1 in epidermal keratinocytes were further confirmed to be decreased in a dose-dependent manner.

4. Discussion

The process of melanosome transfer has been investigated for decades either by co-culturing epidermal kerati-

nocytes with melanocytes or incubating epidermal keratinocytes in the presence of isolated melanosomes mostly from melanoma cells. Although several mechanisms of melanosome transfer are likely to occur in the skin, recently, a new mechanism of melanosome transfer has been reported that involves the release of melanosome-containing globules and then uptake by keratinocytes [10]. However, it has been found that the removal of melanosomal membranes due to the detergent treatment during the purification process did not block the transfer into keratinocytes, suggesting that the melanosomal membrane is not critical for the ingestion by keratinocytes.

In the present study, we demonstrated that keratinocytes could uptake melanin directly into cytoplasm. Melanin alone in cytoplasm could also be accumulated to perinuclear site that did not require the components from melanosome. The direct uptake of melanin alone could also be inhibited by trypsin inhibitor and niacinamide, indicating the involvement of protease-activated receptor-2 (PAR-2) in this process, which was similar to previous studies using co-cultured cell model or incubation with isolated melanosomes.

Compared to the previous studies using co-cultured cell model or incubation with isolated melanosomes, this simplified cell model for direct uptake of melanin can be used to investigate the pure effect of melanin on the biological activity of epidermal keratinocyte without being interfered by some unknown factors in the melanosomes particularly those isolated from cancerous melanoma cells instead of normal cells. This cell model was apparently much easier and could avoid the experimental complication due to the different preparation of melanosomes and therefore data with higher reproducibility could be obtained.

Melanin plays an important role in protecting human skin from the UV irradiation. Nuclear accumulation forms a melanin cap above cell nuclei and functions to protect DNA from UV-induced damage. The uptake of melanin in keratinocytes resulted in the inhibition of cell proliferation not only in cultured epidermal keratinocytes, but also being observed in human solar lentigo tissue. The inhibition of cell proliferation is consistent to the result of delay of cell cycle, which may allow the cells to repair the potential damage of DNA and prevent the change of DNA sequence that could be fixed after the replication of DNA in keratinocytes. A recent study reported an inhibited cell proliferation of SV40T-transformed human epidermal keratinocytes (SV-HEKs) after the treatment with isolated melanosomes also in a PAR-2 dependent manner [17]. It is very likely that the inhibition of cell proliferation was caused by the melanin that got inside the cells through the uptake of melanosomes. In addition, based on the results of our microarray results, melanin was indeed found to down-regulate the expressions of many cell cycle-dependent genes in epidermal keratinocytes that contributed to the inhibition of cell proliferation.

Dickkopf 1 (DKK1), an inhibitor of Wnt signaling, not only functions during the embryonic development [18], but also regulates joint remodeling [19] and bone formation [20]. DKK1 is therefore believed to play a role in the pathogenesis of rheumatoid arthritis [21] and multiple myeloma [22]. Recently, the levels of DKK1 in palmoplantar dermal fibroblasts were found to be higher than those observed in non-palmoplantar dermal fibroblasts [23]. In a paracrine signaling way, DKK1 secreted from fibroblasts can suppress melanocyte function through the regulation of microphthalmia-associated transcription factor (MITF) and beta-catenin [24]. In addition, DKK1 can also induce the expression of keratin 9 and alpha-Kelch-like ECT2-interacting protein (alphaKLEIP) but decrease the expression of beta-catenin, glycogen synthase kinase 3beta, protein kinase C, and proteinase-activated receptor-2 (PAR-2) in epidermal keratinocytes which is consistent with the expression pattern in human palmoplantar skin [25]. More interestingly, treatment with exogeneous DKK1 could result in the hypopigmentation and thickening of a reconstructed skin model, elucidating why human palmoplantar skin is thicker and paler than non-palmoplantar skin [26]. Therefore, DKK1 can potentially be used to reduce skin pigmentation or to repair certain damaged skins. Our study, for the first time, demonstrated that keratinocytes can also produce DKK1 by themselves and the DKK1 gene expression in epidermal keratinocytes could be down-regulated by the uptake of melanin. Also, the decrease of DKK1 by melanin uptake was correlated well with the inhibited proliferation of epidermal keratinocytes. In other words, the uptake of melanin in epidermal keratinocytes might play a crucial role in the process of pigmentation that is likely mediated by the suppression of DKK1 gene expression. These interesting results warrant further extensive investigation.

5. Conclusion

In conclusion, our findings demonstrated a cell model that was potentially useful to study the effects of melanin uptake in epidermal keratinocytes; in addition, our findings demonstrated that after melanin uptake, keratinocyte cell cycle is delayed, which is consistent to the observation that in the solar lentigo area, the cell proliferation is

inhibited. The discovery that DKK1 gene expression in this cell model was down-regulated by melanin uptake further confirmed the physiological meaning of this cell model which could be used to quickly screen drugs that might potentially result in hyper- or hypopigmentation. This simplified cell model could also be useful for exploring the molecular mechanism of melanin uptake and how melanin impacts keratinocyte biological activities, in addition, except DKK1, target proteins of skin pigmentation could be identified and help us to further understand the skin pigmentation issue.

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Conflict of Interests

The authors declared no conflicts of interest.

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