Development of Tyrosinase Promoter-Based Fluorescent Assay for Screening of Anti-melanogenic Agents

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For screening of skin-whitening ingredients that modulate inhibition of melanogenesis, tyrosinase promoter-based assay using a three-dimensional (3D) spheroid culture technique is a beneficial tool to improve the accuracy of raw material screening in cosmetics through mimicking of the in vivo microenvironment. Although the advantages of high-throughput screening (HTS) are widely known, there has been little focus on specific cell-based promoter assays for HTS in identifying skin-whitening ingredients that inhibit accumulation of melanin. The aim of this study was therefore to develop a large-scale compatible assay through pTyr-EGFP, an enhanced green fluorescent protein (EGFP)-based tyrosinase-specific promoter, to seek potential melanogenesis inhibitors for cosmetic use. Herein, a stably transfected human melanoma cell line expressing EGFP under the control of a 2.2-kb fragment derived from the tyrosinase gene was generated. Spontaneous induction of the tyrosinase promoter by 3D spheroid culture resulted in increased expression of EGFP, providing a significant correlation with the tyrosinase mRNA level, and subsequent inhibition of tyrosinase activity. Importantly, the pTyr-EGFP system provided successful tracking of the changes in the live image and real-time monitoring. Thus tyrosinase promoter-based fluorescent assay using a 3D spheroid culture can be useful as a screening system for exploring the efficiency of anti-melanogenesis ingredients.

Key words three-dimensional spheroid culture; tyrosinase promoter; fluorescent assay; high-throughput screening (HTS)

MATERIALS AND METHODS

Cell Culture and Ultra Low Attachment Cell Culture
A375 human melanoma cells (KTCC: No.80003) were grown in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone, Logan, UT, U.S.A.) supplemented with 10% fetal bovine serum (FBS), penicillin (100 unit/mL), and streptomycin (100 µg/mL). The cells were grown at 37°C with 5% CO₂. Cell passage was able to be carried out with a single cell to keep the cells alive and extend the cell growth under cultured conditions. Cells should be passaged when reaching 70–80% confluence. A375-pTyr-EGFP cells were cultured in Ultra Low Attachment (Corning, Corning, NY, U.S.A.) and adherent 96-well plates (NUNC, Rochester, NY, U.S.A.) at a density of 1×10⁵ cells/well with 1 mL of 10% FBS DMEM for 72 h. Imaging analysis was performed with a TCH-5.0 ICE camera (TU CSEN, China) by ECLIPSE TS100 (Nikon, Japan).

Generation of pTyr-EGFP Construct and Transfection
The tyrosinase promoter was removed from the pTyro reporter construct38 by XhoI and HindIII digestion. A 2.2-kb region of the tyrosinase promoter was cloned into a plasmid vector containing the coding region of the enhanced green fluorescence protein (EGFP) gene in the pEGFP-1 vector (BD Biosciences, Palo Alto, CA, U.S.A.) harboring the neomycin resistance gene under the control of the SV40 early enhancer. The final constructs were sequenced completely for confirmation. A375 cells were transfected with the pEGFP-tyrosinase promoter (pTyr-EGFP) linearized by XhoI digestion for random insertion using lipofectamine LTX and PLUS Reagent, according to the manufacturer’s instructions (Invitrogen, San Diego, CA, USA).
U.S.A.). At 24 h posttransfection, neomycin (Sigma, St. Louis, MO, U.S.A.) was added to the cell culture media for 2 weeks to select cells containing the neomycin resistance gene. The medium was changed every third day. After the 2-week period for selection, the cells were harvested at 70–80% confluence and cryopreserved.

Fluorescence-Activated Cell Sorting of Tyrosinase-EGFP Positive Cells and Analysis A375-pTyr-EGFP cells were cultured in 96-well microplates and Ultra-Low Attachment 96-well plates at a density of 1×10^4 cells/well in the appropriate growth medium, with or without cocoon (100 ppm), Hydroquinone (HQ) (5 µM), and Arbutin (Arb) (300 µM). After incubation for 72 h, single cell suspensions of A375-pTyr-EGFP cells were generated using Trypsin (Gibco, Grand Island, NY, U.S.A.). Spheroid aggregates and adherent cells were sorted using a 40 µm Nylon cell strainer (BD Biosciences, Bedford, MA, U.S.A.). For detection of live/dead cells, 1×10^5 cells were incubated with 10 µM of propidium iodide (Immuno Chemistry Technologies, Bloomington, MN, U.S.A.) for 30 min. Single cells were suspended in phosphate buffered saline (PBS) at a concentration of 10^5 cells/mL. Cells were washed again three times and resuspended in PBS before subjected to flow cytometry using an AccuriC6 FACS with BD Accuri C6 Software (BD Biosciences, Palo Alto, CA, U.S.A.) for quantitation of the EGFP expression between groups. Sorting was performed using a Becton Dickenson FACS Aria (BD Biosciences, San Diego, CA, U.S.A.). EGFP positive and negative cells were sorted using the FL1 channel at 224 events/second and low sheath pressure through a 100 µm sort nozzle. Sorted cells were pelleted by centrifugation (1000 rpm for 5 min) and then used for subsequent applications.

RNA Preparation and Real-Time Polymerase Chain Reaction (PCR) Analysis Total RNA was extracted from the A375-pTyr-EGFP cells using the RNaseasy Mini kit (Qiagen, Chatsworth, CA, U.S.A.), according to the manufacturer’s instructions. Single strand cDNA was prepared from 1 µg of RNA using the PrimeScriptII 1st strand cDNA Synthesis kit (TaKaRa, Shiga, Japan), and each PCR amplification was performed using the following primers: GAPDH (5′- TGCCATCGTGAGATGGTGG-3′) and 20 fields of each well were taken. There were no effects of these treatments on the expression of GAPDH, confirming the quality of RNA extraction and reverse transcription. A375-pTyr-EGFP cells were plated at 3×10^4 cells/well in the appropriate growth medium with or without cocoon (100 ppm), Hydroquinone (HQ) (5 µM), and Arbutin (Arb) (300 µM) for 72 h. The cells were washed twice with PBS and lysed with radioimmunoprecipitation assay (RIPA) buffer. A substrate solution (5 µl of 10 mM 7,3,4-dihydroxyphenylalanine (l-DOPA)) was then added to each well, after which the supernatants were collected and incubated at 1 h at 37°C. The tyrosinase activity of each sample was determined based on the optical density at 470-nm using a spectrophotometer. The final tyrosinase activity of each sample was normalized using a control for each sample. The experiments were repeated at least three times.

Image Analysis of pTyr-EGFP Positive Cells A375-pTyr-EGFP cells were cultured in 96-well microplates and Ultra-Low Attachment 96-well plates at a density of 1×10^4 cells/well in the appropriate growth medium with or without cocoon (100 ppm), Hydroquinone (HQ) (5 µM), and Arbutin (Arb) (300 µM) for 24–72 h. For image acquisition, the plates were loaded into an IN Cell Analyzer 1000 (GE Healthcare, Franklin Lakes, NJ, U.S.A.). Images of individual wells were automatically acquired by IN Cell Analyzer 1000, which generated fluorescence microscopy images of the cell samples in a multwell plate format in an automated manner, followed by automated image analysis. From each well of the plate, 4 z-series were scanned from top to bottom, and 20 fields of each well were taken. There were no effects on the development of A375-pTyr-EGFP cells during this process. The intensity of each cell was identified using the background fluorescence of EGFP expression (EGFP, excitation 475-nm; emission 535-nm). Images were analyzed using IN Cell Investigator software.

Statistical Analysis Each experiment was performed at least three times, and the results were reported as mean±standard deviation (S.D.). Significant differences between results were determined by Student’s t-test and Microsoft Excel. A p-value <0.05 was considered significant.

RESULTS AND DISCUSSION

Correlation between Tyrosinase mRNA Expression and Tyrosinase Promoter Activity To exploit the activity of melanogenesis induced by the spheroids for image-based screening, a fusion gene harboring the 2.2-kb 5′ flanking region of the tyrosinase gene linked to the coding region of EGFP was generated, and a cell line was established (Fig. 1A). A frac-
tion of A375 human melanoma cells weakly expressed EGFP, which could be isolated by fluorescence-activated cell sorting (Fig. 1B). Noticeably, only the spheroid formation of melanin-producing cells in the Ultra Low Attachment plate stimulated physiological melanin biosynthesis without the addition of α-MSH (unpublished data). To examine melanin biosynthesis by the 3D spheroid culture in the Ultra Low Attachment cell culture plates, A373-pTyr-EGFP cells were cultured in both 2D culture and 3D spheroid culture for 72 h. In the 3D spheroid culture, significant increase of the melanin content and number of EGFP-positive cells was observed compared with the 2D culture (Fig. 1C). For quantification, the EGFP-positive cells in spheroids were counted and correlated to the number of melanin-accumulating cells by FACS analysis. The EGFP-positive cells in the 3D spheroid culture showed a significant increase (30.4%) compared with the 2D culture (0.2%) (Fig. 1D). To examine the correlation between the expression efficiency of tyrosinase mRNA in the 2D culture and 3D spheroid culture, the amount of tyrosinase mRNA was determined by real-time PCR. The levels of tyrosinase mRNA expression were significantly increased in the 3D spheroid culture (Fig. 1E). These results demonstrated the efficacy of the pTyr-EGFP construct stimulated in the 3D spheroid culture, which showed a correlation between the tyrosinase mRNA expression and tyrosinase promoter activity.

**Effect of 3D Spheroid on Cell Viability and Melanogenesis** To investigate the effect of 3D spheroid culturing on cell viability and melanin biosynthesis, A375-pTyr-EGFP cells were cultured in both Ultra Low Attachment and adherent 96-well plates for 72 h. The cell viability was measured by the MTT assay. For melanin content, the same number of cells was harvested for visual evaluation of the different melanin contents, and the pellets were subsequently incubated in 1 N NaOH for 90 min at 60°C. The supernatant was measured at 450-nm using a spectrophotometer. As shown in Fig. 2, the 3D spheroid culture showed a significant reduction in cell proliferation (57.09±2.9%) compared with the 2D adherent culture (100±7.8%) (Fig. 2A). On the other hand, a significant increase of melanin was detected in the 3D culture-stimulated A375 melanoma cells (173.6±11.1%) (Fig. 2B). On the basis of this observation, the 3D spheroid culture protocols resulted in marked increase of melanin biosynthesis while reducing proliferation.

**Correlation between Automated Imaging-Based Screening and FACS Analysis** To establish the assay conditions for imaging-based screening, the effects of depigmentation agents were monitored systematically using automated fluorescence confocal microscopy and FACS. EGFP-positive...
Spheroids were generated using the stable A373-pTyr-EGFP cells, and the 3D spheroids were dispensed in 96-well plates for 72 h (Fig. 3A). Six compounds were tested over a selective range of concentrations for tyrosinase induction and inhibition. EGFP expression under the tyrosinase promoter in the 3D spheroid culture treated with α-melanocyte stimulating hormone (α-MSH (50 nM)) was increased to ca. 2.5-fold higher than in the 2D culture (Figs. 3A, B). In addition, the 3D spheroids treated with vit (1, 3 µM), koj (300, 500 µM), cocoon (100, 500 ppm), Hydroquinone (HQ) (1 µM), and Arbutin (Arb) (300, 500 µM) for 72 h (n=3). (D) Quantification of FACS analysis to obtain the effects of depigmentation agents in A375-pTyr-EGFP cells.

Fig. 3. Live Cell Imaging-Based High-Throughput Screen for Skin-Whitening Ingredients That Inhibit Melanogenesis Triggered by 3D Spheroid Culture

(A) A375-pTyr-EGFP cells were plated in 96-well microplates and Ultra-Low Attachment plates then treated with α-MSH (50 nM), vitamin (vit) (1, 3 µM), kojic acid (koj) (300, 500 µM), cocoon (100, 500 ppm), Hydroquinone (HQ) (1, 5 µM), and Arbutin (Arb) (300, 500 µM) for 72 h. Live cell images of EGFP-positive cells were captured by automated fluorescence microscopy at the indicated times (n=3). (B) Quantitative analysis of the EGFP intensity in EGFP-positive cells. (C) FACS analysis of EGFP-positive cells after treatment with cocoon (100 ppm), Hydroquinone (HQ) (1 µM), and Arbutin (Arb) (300 µM) for 72 h (n=3). (D) Quantification of FACS analysis to obtain the effects of depigmentation agents in A375-pTyr-EGFP cells.
500 µM) did not lead to a dose-dependent reduction of EGFP expression under the tyrosinase promoter in the 3D spheroid culture when compared to the effects of other depigmentation agents. Despite the safety of arbutin as a depigmentation agent, higher concentrations of arbutin may often cause hyperpigmentation (Figs. 3A, B).

To confirm the results obtained with the IN Cell Analyzer 1000 system, 3D spheroids were treated with cocoon (100 ppm), HQ (1 µM), and Arb (300 µM) for 72 h, after which the EGFP expression levels were determined by flow cytometry (Fig. 3C). As expected, the 3D spheroid cells treated with cocoon, HQ, and Arb presented ca. 2–5-fold higher inhibition of the tyrosinase promoter activity compared to the activity of 42.5 ± 1.2% detected for the control (Fig. 3D). These data correlated with the measurement and monitoring of tyrosinase promoter activity by automated fluorescence microscopy.

**Inhibition of Tyrosinase Activity in 3D Spheroid Culture**

The effects of cocoon, HQ, and Arb on cellular tyrosinase activity in 2D culture and 3D spheroid culture were investigated. In the 2D adherent culture, the tyrosinase activity in A375 cells treated with cocoon (100 ppm), HQ (5 µM), and Arb (300 µM) did not show a significant difference compared with the control, while the 3D spheroid culture control (184 ± 2.1%) stimulated ca. 2-fold higher level than that of the 2D control (100 ± 1.3%). Importantly, tyrosinase activity in the 3D spheroid culture treated with cocoon (48 ± 4.7%), HQ (52 ± 2.6%), and Arb (124 ± 9.2%) showed reductions of ca. 2.8, ca. 2.5, ca. 0.5-fold compared to the control level (184 ± 2.1%) (Fig. 4).

A significant positive correlation was found between the tyrosinase activity and the fluorescent activity of pTyr-EGFP. Indeed, HTP techniques using 3D spheroid cultures would provide a more accurate representation of *in vivo* environments, better information to test drug efficacy, and more rapid, specific cellular effects at various targets than 2D cultures.\(^{13}\)

Although HTS assays have been useful for pre-clinical screening, efficacy tests of anti-cancer drugs, and biological relevance,\(^{13}\) the HTP technique using 3D spheroid cultures has not yet been used for the screening of skin-whitening ingredients. However, we demonstrated that 3D spheroid cultures resulted in marked increase of melanin biosynthesis and reduction of proliferation.\(^{10}\)

Here, we presented a new HTS analysis method for screening of skin-whitening ingredients *via* a combination of automated fluorescence microscopy and tyrosinase promoter-based fluorescent assay. For this screen, a stable cell line harboring an EGFP reporter gene under the tyrosinase promoter region was generated, which places the reporter gene in the correct integration for EGFP expression to be driven by the tyrosinase promoter region. Melanin synthesis is mainly regulated by tyrosinase, which is the inhibition target of vit, Arb, koj, cocoon, and HQ, used as cosmetic and medical materials with depigmentation agents.\(^{9–11}\)

To investigate the depigmenting efficacy as shown by a combination of automated fluorescence microscopy and tyrosinase promoter-based fluorescent assay in 3D culture, images were acquired with an automated IN Cell Analyzer 1000 for the purpose of time-lapse acquisition in Ultra-Low Attachment 96-well plates. A combination assay using the IN Cell Analyzer 1000 makes potentially more efficient use of cost, time, and accuracy for the screening of skin-whitening ingredients, resulting in increased assay robustness.\(^{14}\) In addition, 3D spheres applied for microscopy-based measurement can also potentially be verified in the flow cytometry-based assay. Two concentrations were scored per depigmentation agent with three wells per concentration, and 20 fields of fluorescent microscopic images were taken per well. The results demonstrated that four of the depigmentation agents, vit, koj, cocoon, and HQ, showed significant inhibitory effects on the tyrosinase promoter-based fluorescent intensity. However, the efficacy of Arb was rather poorer than the others for inhibition. Thus, Arb (300 µM) only weakly inhibited human tyrosinase activity and subsequent pTyr-EGFP intensity, while vit (3 µM), koj (500 µM), cocoon (100 ppm), and HQ (5 µM) were more powerful than Arb in the 3D culture.

In conclusion, we presented a novel, sensitive, accurate HTS method by using a 3D spheroid culture for the screening of skin-whitening ingredients. This study showed that the combination of the tyrosinase core promoter with the EGFP

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**Fig. 4. The Inhibitory Effects on Tyrosinase Activity in 3D Spheroid Culture**

3D spheroid culture stimulated physiological tyrosinase activity in A375-pTyr-EGFP cells, which were then treated with cocoon (100 ppm), Hydroquinone (HQ) (5 µM), and Arbutin (Arb) (300 µM) for 72 h (n=3). Tyrosinase activity was determined using l-DOPA. The data are presented as the mean±S.E.M., *p*<0.005, **p*<0.0001, ***p*<0.0001.
reporter gene is a powerful assay to constitutively monitor dynamic molecular events in live cells, such as cell morphology, proliferation, increase of melanin contents, and inhibition of melanin production to explore the possibility of induction of melanin biosynthesis. Although cell-based fluorescent screening has the disadvantages that it is unable to identify skin-whitening ingredients which directly inhibit tyrosinase activity, the combination assay system developed herein would be effective for the screening of depigmenting products. It provides a powerful method for high-throughput screening, allowing a better understanding of melanogenesis in in vitro-like 3D spheroid culture to find potential skin-whitening ingredients.

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Conflict of Interest  The authors declare no conflict of interest.

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