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Establishment of a melanogenesis regulation assay system using a fluorescent protein reporter combined with the promoters for the melanogenesis-related genes in human melanoma cells

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ABSTRACT

There are two established depigmenting agent assays currently in use. However, these methods are unreliable and time-consuming. Therefore, it will be valuable to establish a better assay system for depigmenting agent analysis. In this study, we established a melanogenesis regulation assay system using a fluorescent protein reporter combined with the promoters for the microphthalmia-associated transcription factor (MITF), tyrosinase (Tyr) and dopachrome tautomerase (Dct) genes in MeWo human melanoma cells. We used several melanogenesis regulators, including theophylline, hesperetin, arbutin and rottlerin, to confirm the function of this assay system. The established MeWo/pMITF-EGFP, MeWo/pTyr-EGFP and MeWo/pDct-EGFP stable cells integrated the pMITF-EGFP, pTyr-EGFP and pDct-EGFP plasmids into their genomic DNA. These stably transfected cells were used to examine alterations in the expression of the MITF, Tyr and Dct genes. All of the tested compounds, including theophylline, hesperetin, arbutin and rottlerin, could be analyzed in the stable cells, producing reliable results. Therefore, we believe that this melanogenesis regulation assay system can be used as a rapid and reliable assay system to analyze the regulation of melanogenesis by many known or unknown compounds.

1. Introduction

Skin pigmentation arises from the production and distribution of melanin by melanocytes in the epidermal layer, which is the main physiologic defense against harmful ultraviolet (UV) irradiation. However, the over-production of melanin frequently causes hyperpigmentation in the skin [1]. As shown in Fig. 1A, melanin synthesis is initiated with the first step of tyrosine oxidation by tyrosinase (Tyr), which is a copper-containing enzyme that produces melanin by the hydroxylation of tyrosine into dihydroxyphenylalanine (DOPA) and by the oxidation of DOPA into dopaquinone [2]. In the absence of cysteine, dopaquinone gives rise to dopachrome. The dopachrome is then converted to 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA). DHICA production is catalyzed by another important enzyme, dopachrome tautomerase (Dct), also known as tyrosinase-related protein-2. Finally, the dihydroxyindoles are oxidized to produce eumelanin [3]. Tyrosinase and tyrosinase-related proteins (TRPs), including Dct, are transcriptionally regulated by a key transcription factor, microphthalmia-associated transcription factor (MITF) (Fig. 1A) [4,5]. MITF is one of the basic helix-loop-helix leucine zipper (b-HLH-zip) transcription factors. MITF was shown to bind to one or both of the two conserved consensus sequences, the M-box or E-box, in the promoter region of its regulated genes. The tyrosinase and Dct promoters all have at least one M-box consensus sequence (Fig. 1B) [6,7]. Therefore, the inhibition of MITF, Tyr and Dct is the most general approach for achieving skin hypopigmentation. The development of novel, potent and safe depigmenting agents is of great importance in the medical, food and cosmetics industries [8,9].

There are two established depigmenting agent assays currently in use. The in vitro mushroom tyrosinase activity assay is frequently used to discover new tyrosinase inhibitors [10,11]. However, the fungus tyrosinase is quite different from the mammalian tyrosinase. Therefore, this system provides partial reliable results [12]. The cell-based melanin content assay in mouse melanocytes (e.g., B16F10 mouse melanoma cells) is better than the mushroom tyrosinase activity assay because it provides more credible results

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[13,14]. However, this assay is time-consuming, requiring approximately 3–5 days to complete. Moreover, mouse melanoma cells also have some differences from human melanocytes in terms of melanogenesis [15]. Besides, almost all human melanoma cells (e.g., MeWo human melanoma cells) produce a relative insufficient melanin content that results in its difficulty to be achieved from human melanocytes by the melanin content assay. Thus, the establishment of a new assay system would be valuable to melanogenesis research.

Gene regulation is often difficult to observe in cells, unless a reporter gene system is developed to examine the precise gene regulation [16]. Fluorescent proteins are frequently used in reporter gene systems because of their simplicity, rapidity and clarity [17–19]. In this study, we established a melanogenesis regulation assay using a fluorescent protein reporter combined with the promoters for the MITF, Tyr and Dct genes in MeWo human melanoma cells. We used several regulators of melanogenesis, including theophylline, hesperetin, arbutin and rottlerin, to confirm the function of this assay system. The established cell-based melanin content assay in mouse melanoma B16F10 cells is used as a control experiment in this study. Our results indicated that this new system can be used as a rapid and realistic assay system to analyze the regulation of melanogenesis. Moreover, the analysis can confirm the regulation targets of the tested compounds. Therefore, we anticipate that this system can be applied for the development of drugs, foods and cosmetics.

2. Materials and methods

2.1. Materials

Theophylline, hesperetin, arbutin, G418, ethidium bromide, dithiothreitol (DTT), agarose and other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). Rottlerin was purchased from Merck (Hohenbrunn, Germany). Fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin, deoxynucleotide triphosphate (dNTP), oligo(dT), *Pfu* and *Taq* DNA polymerase, M-MLV reverse transcriptase, Modified Eagle Medium (MEM) and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Gibco BRL/Invitrogen (Carlsbad, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Affymetrix/USB (Cleveland, OH, USA). Xfect™ Transfection Reagent was purchased from Clontech Laboratories, Inc. (Mountain View, CA, USA). Restriction enzymes were purchased from New England BioLabs (Beverly, MA, USA). Deionized distilled water (ddH_2O) used to prepare solutions was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Plasmid construction

The genomic DNA from MeWo human melanoma cells was extracted by FavorPrep™ Blood/Cultured Cell Genomic DNA Extraction Mini Kit (Favorgen, Ping-Tung, Taiwan, ROC). Human MITF, Tyr and Dct promoters (Fig. 1B) DNA fragments were amplified through the extracted genomic DNA by *Pfu* DNA polymerase with designed primers (Table 1). The prepared promoter DNA fragments were then cloned into pEGFP-1 (Fig. 1B) vectors (Clontech, Mountain View, CA, USA) by *Xba*I and *EcoR*I restriction sites for MITF and Dct, *Xba*I and *Sac*II restriction sites for Tyr to generate pMITF-EGFP, pTyr-EGFP and pDct-EGFP plasmids (Fig. 1B).

2.3. Cell culture, transfection and stable cell selection

The MeWo cells (BCRC 60540, human melanoma cell line) and B16F10 melanoma cells (BCRC 60031, mouse melanoma cell line) were obtained from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan, ROC). MeWo and B16F10 cells were respectively cultured in MEM and DMEM supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin-streptomycin (100 U/mL penicillin and 100 μg/mL streptomycin). The cells were maintained in a humidified incubator at 37 °C with 5% CO_2. The cells were sub-cultured every 3–4 days to maintain logarithmic growth and were allowed to grow for 24 h before transfection or treatment were applied. For transfection, MeWo cells were cultured in 24-wells plates with 1×10^5 cells/well and the prepared plasmids, pMITF-EGFP, pTyr-EGFP and pDct-EGFP, were transfected by Xfect™ Transfection Reagent into cells at concentration of 1 μg/well according to the protocol. Transfected cells were analyzed by fluorescent microscopy to confirm the protein expression and then further measured by ELISA reader. The transfection efficiency of tested plasmid was measured by the co-transfected control plasmid (pDsRed) at the ratio of 10:1. For stable cell selection, after 48 h transfection, the transfected MeWo cells were selected by G418 at concentration of 1 mg/mL. After 3–4 times sub-culture, the stable transfected MeWo cells were sustained at MEM medium with 0.5 mg/mL G418 to maintain the plasmids in

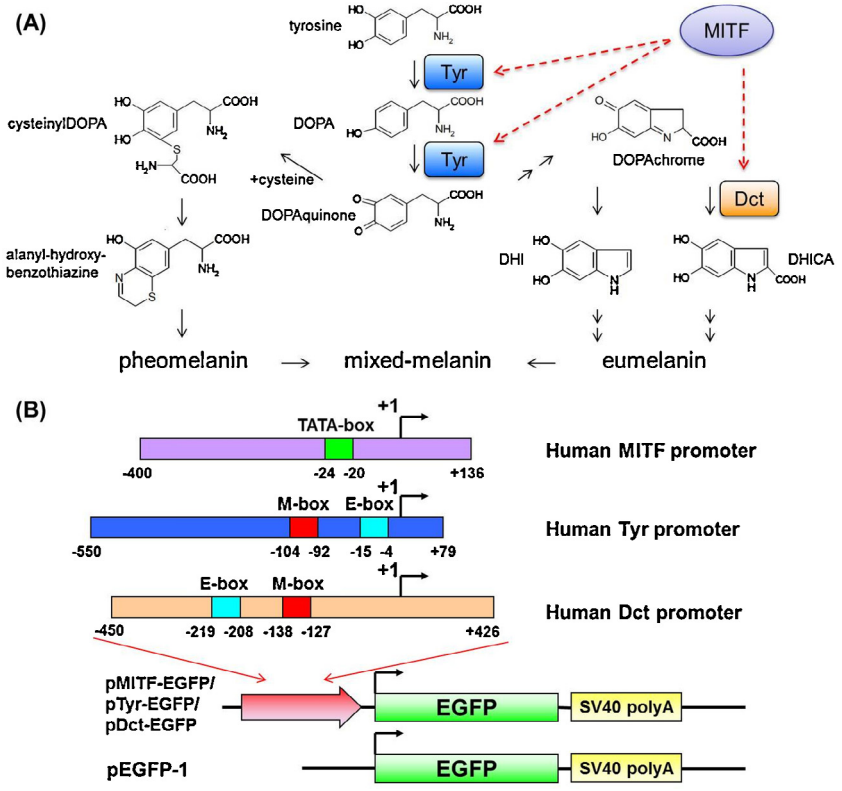

Fig. 1. (A) The schematic diagram of the regulation of melanin production. (B) The human MITF, Tyr and Dct promoters and pEGFP-1, pMITF-EGFP, pTyr-EGFP and pDct-EGFP plasmids.