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ARTICLE

A 2D and 3D melanogenesis model with human primary cells induced by tyrosine

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Abbreviations used: α-MSH, α-melanocyte-stimulating hormone; KBM, keratinocyte basal medium; KGF, keratinocyte growth factor; KGM, keratinocyte growth medium; MC1R, melanocyte-specific melanocortin-1 receptor; MITF, transcription factor associated with microphthalmia; TYR, tyrosinase; TYRP1, tyrosinase-related protein 1

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ABSTRACT

Research on melanogenesis, its regulation in health and disease, and the discovery of new molecules with pigmenting and depigmenting activities use different models. Here we standardize a protocol based on previous ones using primary human melanocytes and keratinocytes in co-cultures, in which melanogenesis was induced under mild conditions by the addition of tyrosine plus ammonium chloride (NH_4Cl). The expression of MITF, TYR, TYRP1, and Melan-A as well as melanin content were measured. Furthermore, we extended this study to a reconstructed 3D model. Pigmentation was visually observable and melanosomes were identified by Fontana-Masson staining by the addition of tyrosine plus NH_4Cl during the stratification phase. The 2D and 3D protocols proposed here circumvent limitations of previous models, using human primary cells and mild conditions for melanogenesis. These protocols offer a viable, robust, simple, and animal-free investigational option for human skin pigmentation studies and screening tests for new compounds that modulate pigmentation.

Keywords: co-culture, keratinocyte, melanin, melanocyte, pigmentation

INTRODUCTION

Skin pigmentation is the result of the melanogenesis process created by the deposition of melanin, a pigment produced by melanocytes and transferred by melanosomes to keratinocytes in response to a stimulus such as UV radiation. The market for the treatment of skin pigmentation disorders is an important economic sector valued at approximately US $5283.82 million in 2017 and is expected to generate a revenue of around US $8479.08 million by the end of 2024 [1]. In this perspective, the identification of new molecules with pigmenting and depigmenting activity is increasingly sought-after by cosmetic and pharmaceutical companies.

In the research and development of skin care products, a variety of experimental models are used. Although skin equivalents such as in-house or commercial 3D models of reconstructed skin are closer to the complexity of normal human skin, there is still a need for simpler and less expensive models for the screening of compounds that can modulate pigmentation.

Melanogenesis is a complex physiological process known to be directly or indirectly regulated by a myriad of distinct genes [2]. The initial step of melanogenesis is the binding of α-melanocyte-stimulating hormone (α-MSH) to the melanocyte-specific melanocortin-1 receptor (MC1R) in melanocytes. This activates adenylate cyclase, leading to elevated levels of intracellular cAMP, which results in an increase in the production of transcription factor associated with microphthalmia (MITF), the major transcription factor in melanogenesis [3]. MITF activates the transcription of tyrosinase (TYR), an enzyme responsible for the hydroxylation of L-tyrosine and consequent trigger of the biochemical cascade of melanin formation. Although the process of melanogenesis has been studied for a long time, there are still gaps in the understanding of how this phenomenon is regulated in health and disease.

Most studies on melanogenesis and its regulation use the murine melanoma B16-F10 cell line because it is easy to grow and to pigment and can be induced by various stimuli such as IBMX, α-MSH, Forskolin, and UV light [4-6]. On the other hand, the fact that they are already modified cells can lead to alterations in the physiological process of pigmentation. Due to the myriad of mutations present in these cells, one should also consider that mutations in genes associated with pigmentation could influence the results and the reliability of conclusions drawn. Other studies used either isolated immortalized human

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melanocytes [7] or co-cultures with keratinocytes [6], which may also lead to artifacts. In the literature, there are studies that used primary melanocyte cultures or co-cultures with keratinocytes but the stimuli used are more aggressive and cause a variety of biological alterations [6,8,9]; other studies used tyrosine as a stimulus but did not focus on the proposal of a protocol for pigmentation tests [10].

Here, we focus on a proposal of a 2D co-culture protocol with human cells originated from donors of different phototypes and with a mild stimulus that does not require irradiation. We choose to induce melanogenesis in a melanocyte-keratinocyte co-culture model with human primary cells using tyrosine plus NH_4Cl as a stimulus. Tyrosine, besides being a substrate for melanin synthesis, stimulates tyrosinase activity, acts synergistically with α-MSH, and is a positive modulator of the MCR1 receptor [11] and NH_4Cl contributes to the melanosome maturation [12]. The pigmentation protocol is applicable in 2D co-cultures and 3D-skin models and uses the content of melanin or the staining of melanosome as endpoints.

MATERIALS AND METHODS

Cell lines and cell coculture

Primary cultures of human melanocytes and keratinocytes were derived from donor's foreskin as previously described [13]. Melanocytes were maintained in 254CF Medium supplemented with Human Melanocyte Growth Supplement (Gibco), and keratinocytes were maintained in keratinocyte basal medium (KBM) Gold Medium supplemented with KBM Gold SingleQuots (Lonza). Contamination control tests for mycoplasma were done weekly. Research Ethics Committee (CEP-HU-USP 943/09).

Co-cultures of keratinocytes and melanocytes were seeded at a ratio of 1:1. 2.5×10^5 keratinocytes were first plated on 100 mm petri plates (Corning®) and after 24 h at 37°C, 2.5×10^5 melanocytes were added in KBM Gold Medium supplemented with KBM Gold SingleQuots. Twenty-four hours after adding the melanocytes, tyrosine (0.1 or 0.25 mM) and ammonium chloride (2 and 5 mM) were added to plates. Co-cultures were incubated for 5 d at 37°C with 5% CO_2 atmosphere in a humidified incubator. The culture medium was not changed during this period.

qRT-PCR-based expression analysis

The total RNA was extracted using the RNeasy Mini kit (Qiagen, Austin, TX, USA) according to the manufacturer's instructions. RNase-free DNase (Qiagen) was used to remove any potential contamination by genomic DNA. The samples were stored at −80°C until use. Up to 1 µg of total RNA was reverse transcribed using the High Capacity RNA-to-cDNA kit (Applied Biosystems, USA), according to the manufacturer's instructions. Real-time PCR was performed by TaqMan fluorescence energy transfer assays with an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Warrington, United Kingdom). The primers and fluorogenic probe used in this assay were obtained from Thermo Fisher and were ordered as Hs00266705_g1 (GAPDH), Hs01117294_m1 (MITF), Hs01099965_m1 (TYR), Hs01060054_m1 (TYRP1), and Hs00194133_m1 (Melan-A). For each real-time reaction, GAPDH was used as an endogenous housekeeping gene. The relative comparison method ($2^{-\Delta\Delta CT}$) was used to compare the expression levels of mRNA.

TYR western blot

Total protein samples were extracted with RIPA buffer (Sigma-Aldrich, St Louis, MO, USA), protease, and proteasome and phosphatase inhibitor cocktail. Total protein (5 µg) was resolved by 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane using the Bio-Rad transfer system (Bio-Rad, Richmond, CA, USA). The membranes were blocked with 5% non-fat-milk in Tris Buffered Saline with Tween 20 (TBST) for 2 h at room temperature with gentle shaking and incubated overnight at 4°C with the primary antibody against TYR (1:1000) (Abcam, Cambridge, UK) (Rabbit recombinant monoclonal Tyrosinase antibody-Lot EPR10141-ab170905). Control of the endogenous proteins was performed at the position of the membrane distant from the proteins of interest with anti-vinculin antibody (1:500) (Mouse recombinant monoclonal anti-vinculin antibody, Cat. # V9131-V9131) (Sigma-Aldrich, Darmstadt, Germany). After washing with TBST, membranes were incubated with a secondary antibody (Sheep anti-rabbit IgG, 1:3000, horseradish peroxidase-conjugated) (Amersham Bioscences, Piscataway, NJ, USA). Membranes were developed using enhanced chemiluminescence reagents (Bio-Rad, Richmond, CA, USA) and the quantification of the bands was performed by the Image J. software.

Melanin content assay

After the incubation of the co-cultures with the melanogenesis inducers, cells were counted and resuspended (1×10^6) in 250 µl of 1 M NaOH with 10% DMSO (extraction solution) and kept at 95°C for 1 h. Following this, 200 µl were transferred to 96-well plates, and absorbance was read at 470 nm. Melanin concentration was calculated from a standard curve for synthetic melanin (Sigma®).

3D skin models

The skin was prepared in two steps as previously described [14]. Briefly, the dermal compartment was prepared using rat tail type-I collagen gel (Corning, Tewksbury, MA) and 1.5×10^5 Fibroblasts per construct. After polymerization of the collagen gel for 2 h, 2.5×10^5 keratinocytes and 1.7×10^5 melanocytes were seeded on top of each construct and were kept submerged in RAFT: KGM-Gold Bullet Kit Medium (1:1) for 24 h. The inserts were then raised and maintained at the air-liquid interface for 12 d for the stratification of the skin. The 3D skin was maintained in a 5% CO_2 incubator with low humidity (~50%), and the medium supplemented with tyrosine (0.25 mM) + NH_4Cl (5 mM) was replaced every 2 d.

Pigmented or unpigmented skin was fixed in 10% buffered formalin at 4°C for 12 h, followed by dehydration and cleaning in solutions containing increasing concentrations of alcohol and xylene for paraffin inclusion. Paraffin sections (5 µm) were stained with hematoxylin and eosin (H&E) for morphological analysis and Fontana-Masson for melanin content analysis. All images were obtained by optical microscopy (Olympus Life Science-40×) and analyzed using NIS-Elements software (Nikon Instruments, Melville, NY).

Statistics

Statistical analysis was performed with GraphPad Prism for Windows, version 5.0 (GraphPad Software, Inc., La Jolla, CA, EUA). The statistical significance of differences in the mean values of all experimental groups was calculated using ONE Way ANOVA and Student's *t*-test. P values < 0.05 were considered to be statistically significant.

RESULTS

The treatment of monolayer melanocytes with 0.25 mM tyrosine and 5 mM NH_4Cl was not able to induce melanin synthesis even in the presence of 20 ng/ml of keratinocyte growth factor (KGF), an inducer of TYR enzyme (**Fig. 1A**) [14]. On the other hand, melanogenesis was induced in co-cultures of keratinocytes and melanocytes using the same conditions as above.

Given the different supplemental needs of keratinocytes and melanocytes in culture, and the fact that in the literature studies using co-cultures take advantage of the keratinocyte medium [15], we compared the morphology and cell growth of melanocytes in three different culture media: 254CF Medium (specific for melanocytes), keratinocyte growth medium (KGM) Gold Medium (specific for keratinocytes), and a 1:1 mixture of these two media (**Fig. 1B**). Although melanocytes proliferation decreased in KGM Gold Medium, we chose to maintain the co-cultures in this medium because melanocyte morphology and cell adhesion was better preserved.

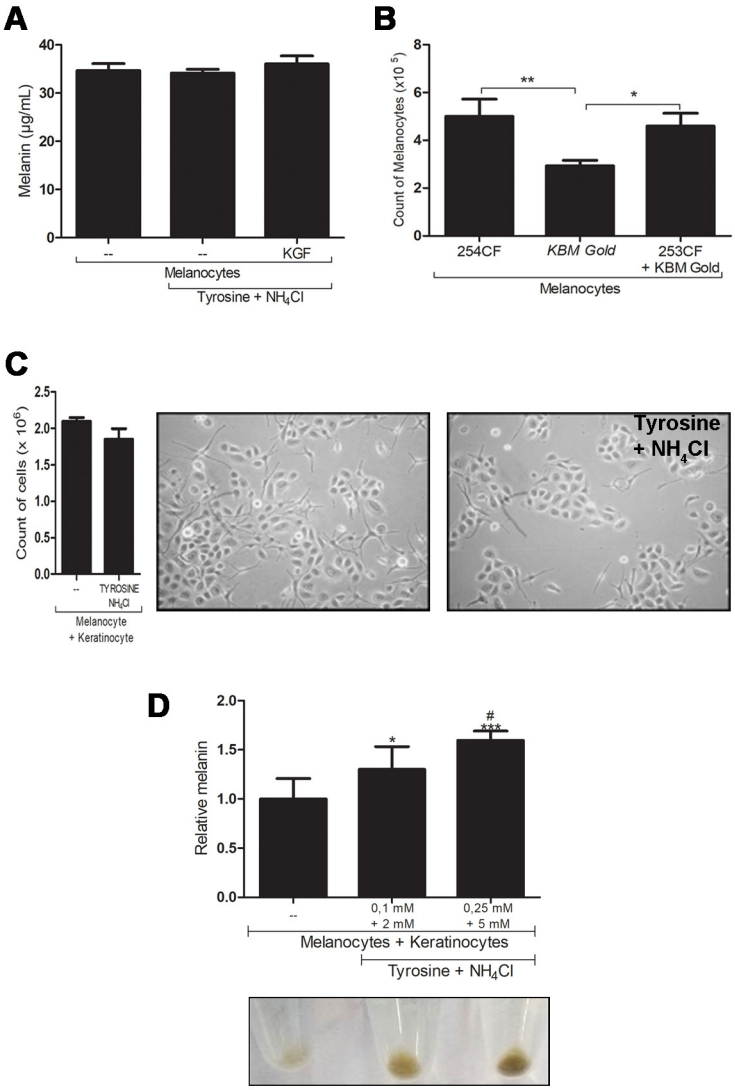

Figure 1. The melanogenesis model using melanocytes plus keratinocytes. A. Melanin content (µg/ml) in melanocytes treated or not with tyrosine (0.25 mM) + NH_4Cl (5 mM) in the presence and absence of KGF (20 ng/ml) in 254CF medium for 5 d (n = 6). **B.** Melanocytes count after 5 d in 254CF, KGM Gold, or 1:1 254CF:KGM Gold medium (n = 3). **C.** Melanocytes + keratinocytes count and microscopy (40x) in co-culture stimulated with tyrosine (0.25 mM) + NH_4Cl (5 mM) for 5 d (n = 6). **D.** Melanin content in melanocytes plus keratinocytes cocultures (2.5 × 10^5 cells each) in presence of tyrosine (0.1 and 0.25 mM) and NH_4Cl (2 and 5 mM) after 5 d and pellets aspect for each condition (n = 6). *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the control. $^{\#}P$ < 0.05 compared with the condition of lowest concentration.

Once the medium was defined, different melanocytes-keratinocytes ratios (1:1, 1:3, 1:5, and 1:10) were tested with the 1:1 ratio offering the best condition. After 5 d of incubation, adequate cell viability (> 90%) and melanin production were observed (**Fig. 1C**). At 0.1 mM tyrosine plus 2 mM NH_4Cl, melanin production increased by about 25%, while the increase was about 50% when 0.25 mM tyrosine and 5 mM NH_4Cl was used (**Fig. 1D**). These increases were also observed by naked eye cell pellet analysis (**Fig. 1D**). Tyrosine plus NH_4Cl, at 0.25 mM and

5 mM respectively, were the concentrations used thereafter. The data represents experiments done with different pools of cells. These pools were composed of melanocytes and keratinocytes from donors with varying pigmentation status with melanin content ranging from 30 to 58 µg melanin/1.10^6 cells. For this reason, melanin concentration was expressed as relative melanin content, comparing basal melanin with melanin measured after the tyrosine + NH_4Cl treatment. Most importantly, regardless of the cell pool used, tyrosine + NH_4Cl was effective in triggering pigmentation, demonstrating that this protocol is robust and can be applied to different compositions of phototype cells.

The gene expression of the four markers of melanogenesis was measured at 8 h, 24 h and 5 d after treatment. At 8 h, MITF, TYR, TYRP1 and Melan-A expression were increased by 2, 1.8, 2.5, and 1.5 times respectively (**Fig. 2**). At 24 h, no differences in the mRNA levels were observed, while at 5 d, a new increase in TYR and an almost total absence of Melan-A expression were observed. TYR expression increased by about 3 times in 48 h of incubation (**Fig. 3A**) and melanin content increased with time, doubling at day 5 (**Fig. 3D**).

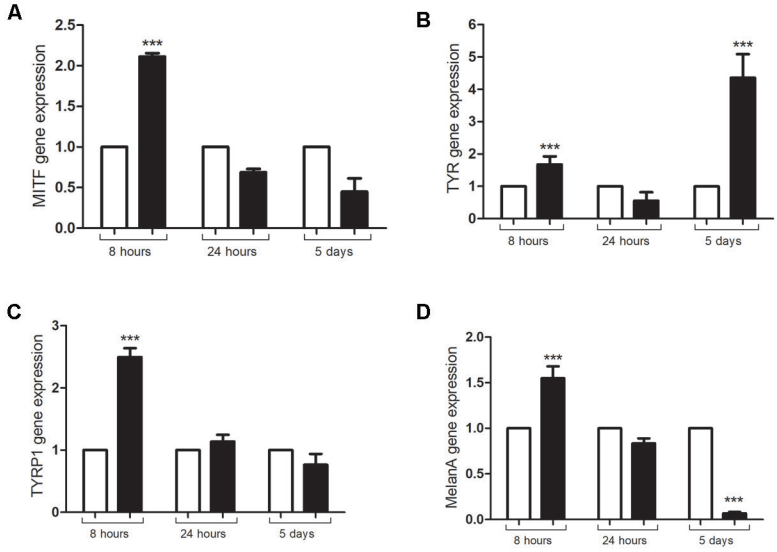

Figure 2. Gene expression of melanogenesis markers. Gene expression of MITF (**A**), TYR (**B**), TYRP1 (**C**), and Melan-A (**D**) in melanocytes plus keratinocytes co-cultures (2.5 × 10^5 cells each) stimulated with tyrosine (0.25 mM) and NH_4Cl (5 mM) for 8 h, 24 h, and 5 d (n = 6). GAPDH was used as the reaction calibrator. Comparative method ($2^{-\Delta\Delta CT}$) was used to analyze the levels of mRNA expression. The white bars refer to the control, and the black bars refer to conditions stimulated with tyrosine plus NH_4Cl. ***P < 0.001.

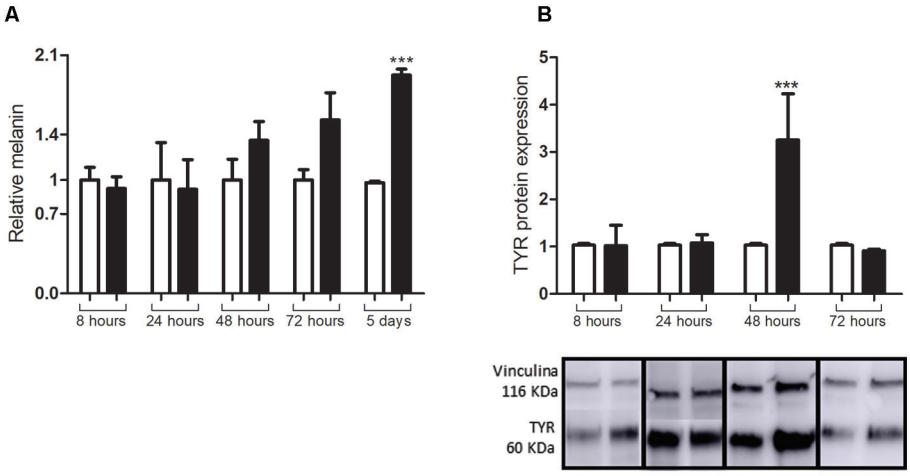

Figure 3. Protein expression of TYR and melanin content over time in melanocytes plus keratinocytes co-culture (2.5 × 10^5 cells each) stimulated with tyrosine (0.25 mM) and NH_4Cl (5 mM) for 8, 24, 48 and 72 h. A. Relative melanin content from 8 h to 5 d (n = 6). **B.** Relative intensity of TYR bands and representative western blot. The endogenous antibody used was anti-vinculin (n = 4). The white bars refer to the control, and the black bars refer to the conditions stimulated with tyrosine plus NH_4Cl. ***P < 0.001.

In order to verify the stimulus of tyrosine + NH_4Cl in a more complex model, we used a lab 3D skin model. During the skin stratification

phase, that is 12 d at the air-liquid interface, 0.25 mM tyrosine and 5 mM NH_4Cl were added in the fresh culture medium every 2 d. Pigmentation was visually observable, and the melanosomes were identified by histological sections stained with Fontana-Masson (**Fig. 4**).

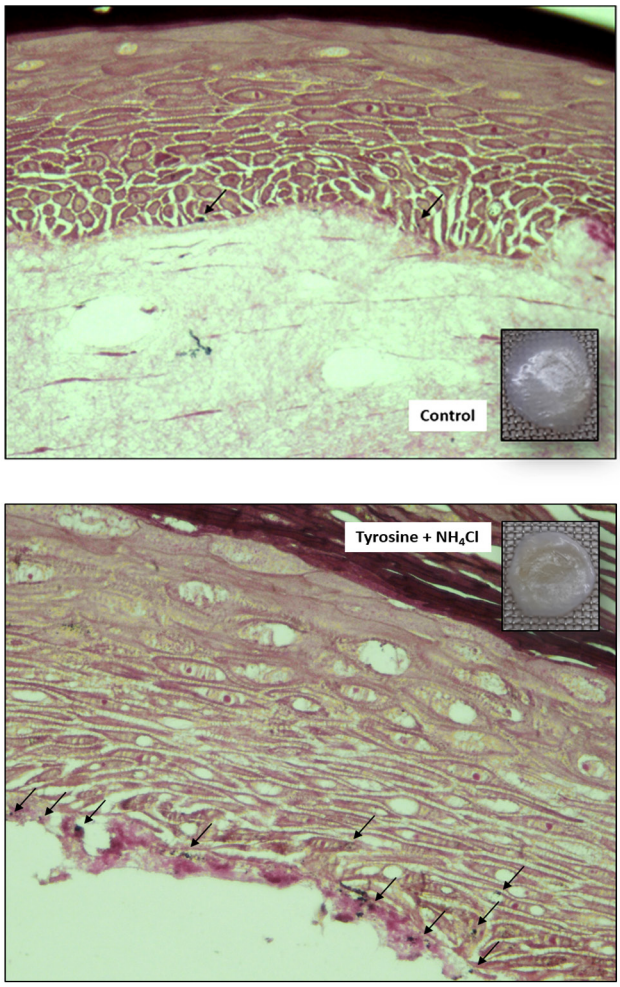

Figure 4. 3D skin model treated with tyrosine (0.25 mM) + NH_4Cl (5 mM). Pigmentation was analyzed macroscopically and on histological sections stained with Fontana-Masson. The arrows indicate the melanin granules in the basal layer of the epidermis.

DISCUSSION

We have demonstrated that co-cultures of melanocytes and keratinocytes treated with tyrosine plus NH_4Cl are able to drive melanogenesis, activating MITF, TYR, TYRP1, and Melan-A and leading to the formation of melanin in sufficient amounts to be measured and visually observed. Under the mild conditions we used, melanocytes alone were not sufficient to drive melanogenesis. Possibly, keratinocytes are needed for melanocyte maturation allowing enough differentiation for a high rate of melanin synthesis [16]. In addition, the presence of keratinocyte ensures the transfer of melanosome from the melanocyte that can feed back the synthesis of more melanin. [17]. Here, we are able to achieve a good response in the production of melanin under mild conditions that can easily be conducted in the laboratory.

The difference in our protocol for inducing melanogenesis by the addition of tyrosine plus NH_4Cl is that it does not require specific equipment such as solar irradiators used in many of the melanogenesis protocols. Although solar radiation is a major trigger of melanin production and it is necessary to mimic solar light to study skin damage, the use of common irradiators have limitations for studies of the control of melanogenesis induced by other stimuli in more physiological conditions. The intensity of artificial irradiation required to promote melanin production usually causes cell damage due to the high dose of energy required during the experiment and the need for cells to remain in culture for several days [18,19]. In our protocol, besides the ease of induction, the inconvenience of sample heating and variations in the irradiation intensity common in most irradiators were avoided.

Besides irradiation, another widely used inducer of melanogenesis is 3-isobutyl-1-methylxanthine (IBMX), which performs this function as an inhibitor of the phosphodiesterase enzyme, increasing intracellular cAMP and thus inducing MITF. IBMX has been described as an inducer of melanogenesis in reconstructed human skin models [20], in immortalized murine melanocytes (Melan-A cell) [21], and in B16-F10 melanomas [22]. IBMX is considered the main positive control for the discovery of compounds with pigmentation inhibiting activity. Despite presenting satisfactory results regarding melanogenesis induction,

IBMX can also generate a variety of cellular alterations such as metabolic or morphological changes [4]. In the cell type used in this paper, IBMX stimulation resulted in large changes in primary cell morphology (data not shown). Forskolin (an activator of adenylyl cyclase) is also frequently used as a pigmentation inducer and may cause a variety of cellular alterations [23].

As our protocol includes and depends on the presence of melanocytes and keratinocytes, it also offers the possibility of studying the cross-talk between these cells. The need for the intense crosstalk between melanocytes and keratinocytes was evident considering that a cell ratio of 1:1 was necessary in order to observe melanin. Keratinocytes produce factors necessary to conduct melanogenesis such as α-MSH, TGF-β, SCF, endothelin, ACTH, granulocyte-macrophage colony-stimulating factor (GM-CSF), besides being protagonists in melanosome transfer [24]. Thus, this model could offer more comprehensive and detailed possibilities of melanocyte and keratinocyte interaction for melanin production.

The increased supply of tyrosine allows analysis of melanin production within 5 d. Without this stimulus, relying solely on co-culture, pigmentation would take longer, *i.e.*, more than 10 d [25]. As already mentioned, tyrosine stimulates TYR activity, acts synergistically with α-MSH, and acts as a positive modulator of the MCR1 receptor [10]. These effects were previously described in mouse melanocytes, in which L-tyrosine restored pigment activity by correctly targeting tyrosinase and TRP1 to melanosomes as well as increasing the number of melanosomes in the final stages of maturation [26]. Similar effects of tyrosine have also been observed in mouse melanocytes with a loss-of-function mutation in MC1R [27], mouse melanoblasts [26], and human melanoma [28].

The present protocol offers a model for melanogenesis with human cells with the possibility of functioning with cells originated from donors of different phototypes and with a mild stimulus. Our study combines conditions that have already been addressed separately in previous studies. No previous study has used co-cultures of primary cells with mild stimuli and measured melanin content and expression and activity of important melanogenic markers within a relatively short time frame.

The use of tyrosine plus NH_4Cl to activate primary melanocyte and keratinocyte co-cultures with melanin content as an endpoint, offers a viable, robust, and relatively simple option for human skin pigmentation studies and for the screening of molecules that modulate pigmentation. Furthermore, incremental pigmentation can be observed macroscopically while the increased number of melanosomes can be microscopically observed by Fontana-Masson staining in a 3D human skin model.

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