hTERT Immortalized Neonatal Melanocytes – an Advanced In Vitro Cell Based Model for Pigmentation and Toxicity Studies

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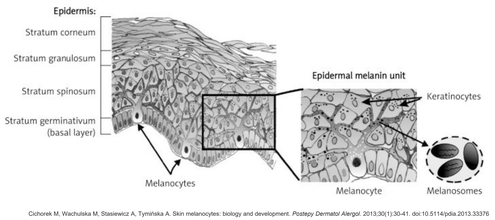
Abstract

Epidermal pigmentation involves two main steps. First, melanocytes perform a myriad of complex biochemical and physiological steps to produce, package, and exocytose melanin-containing melanosomes. Secondly, the melanosomes are taken up by neighboring keratinocytes where the stored melanin protects underlying tissues from damaging UV radiation. This process of pigmentation involves a complex interplay between genetic, endocrine, and environmental factors. Primary cells offer one model system to study pigmentation and dermal agents that may disrupt the melanogenesis; however, they are hindered somewhat by donor-to-donor variability and limited lifespan. Here, we created an immortalized melanocyte cell model—hTERT neonatal melanocytes—by retroviral transduction of human telomerase (hTERT) into primary cells. In addition to enhanced longevity (up to 35 doublings), physiologic marker expression (tyrosinase positive, fibroblast marker negative), and ability to create melanosomes in 3D organotypic co-cultures, the cell line also showed expected levels of responses to several stimulators and inhibitors of melanogenesis. The inhibitors hydroquinone and kojic acid showed dose-dependent decreases in melanin content and the stimulators stem cell factor and latanoprost showed a muted response. In summary, immortalized melanocytes provided a versatile in vitro cell model for the study of skin toxicology and melanogenesis regulation.

Background



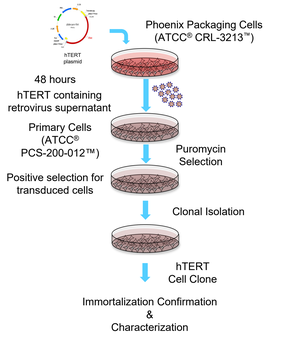
Cichorek M, Wachulska M, Stasiewicz A, Tyminska A. Skin melanocytes: biology and development. *Postepy Dermatol Alergol*. 2013;30(1):30-41. doi:10.5114/pdia.2013.33376

Figure 1. Why Melanocytes? Melanocytes reside in the basal layer of the epidermis and produce protective melanin pigments through a multi-step process. These pigments are packaged into melanosomes, exocytosed, and taken up by neighboring keratinocytes. Models are needed to understand the complex biochemical process and environmental factors that can alter melanin production.

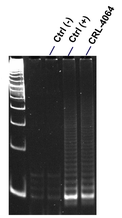
Results

Generation of Immortalized Neonatal Primary Melanocytes

A. Retroviral transduction of primary neonatal melanocytes and clone selection



B. TRAP Assay



C. Diploid Karyotype

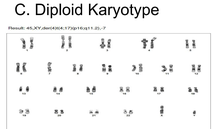
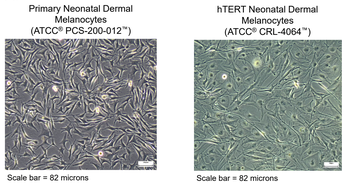


Figure 2. Establishment of hTERT- immortalized neonatal melanocytes. hTERT-immortalized neonatal melanocytes were created by retroviral transduction of the hTERT gene into primary cells. (A) Transduced cells were submitted to puromycin selection and further selected by single cell cloning. (B) High telomerase activity was observed via telomerase reverse transcriptase activity protocol (TRAP assay). (C) Cells maintain a near diploid karyotype.

Characterization of Immortalized Neonatal Melanocytes

A.



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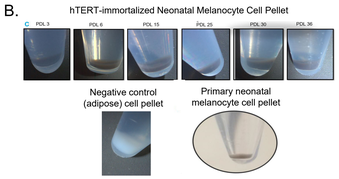

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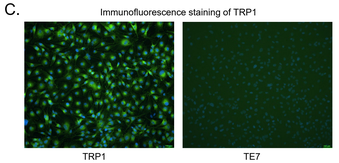

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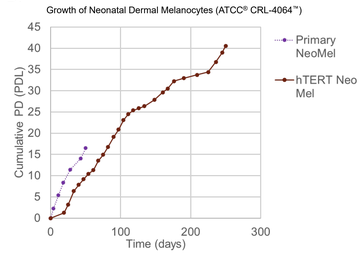
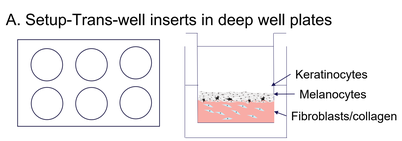


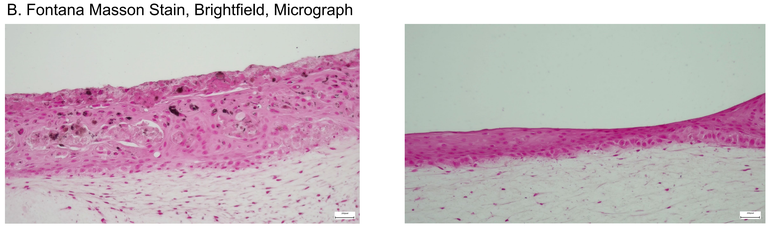
Figure 3. Characteristics of Immortalized Melanocytes. (A) hTERT melanocytes show similar multidendritic morphology as primary melanocytes, and (B) highly pigmented cell pellets indicate melanin is expressed throughout passaging up to PDL 36. (C) Immunofluorescence staining shows immortalized melanocytes express melanocyte specific marker TRP1 and are negative for the expression of fibroblast marker TE-7. (D) Immortalized neonatal melanocytes were continuously passaged for 200 + days (over 40 population doublings) and showed no sign of senescence or cell aging. Primary cells showed decreased growth after 16 PDLs.

Establishment of 3D Organotypic Skin Co-Culture

A. Setup-Trans-well inserts in deep well plates



B. Fontana Masson Stain, Brightfield, Micrograph



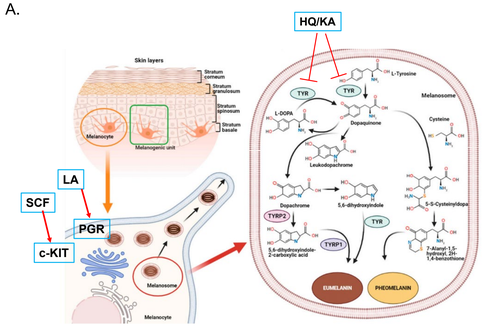
Fibroblast/Keratinocytes + hTERT Neonatal Melanocytes

Fibroblast/Keratinocytes

Figure 4. Integration of immortalized melanocytes into 3D skin co-culture. hTERT-immortalized BJ-5ta (ATCC® CRL-4001™) fibroblasts were embedded into a rat collagen matrix. (A) hTERT-immortalized Ker-CT (ATCC® CRL-4048™) keratinocytes, with or without hTERT melanocytes, were added to the apex of the collagen matrix. After 12 days, cultures were fixed, and matrices were embedded in paraffin and sectioned. (B) Cells were stained using the Fontana Masson method. Staining shows clear melanin deposition throughout the epidermal layer in cultures that contain melanocytes, and no staining in cultures without melanocytes. Additionally, we observed more robust tissue growth in melanocyte containing cultures, suggesting that melanocytes produce additional factors that aid in tissue growth and formation.

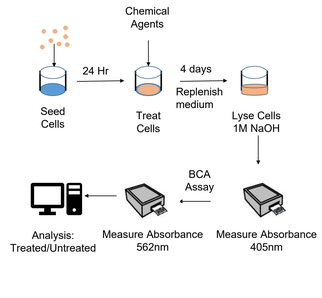
Dose-dependent Response of Melanin Production in Immortalized Melanocytes

A.



Hushcha Y, Blo I, Oton-Gonzalez L, Mauro GD, Martini F, Tognon M, Mattei MD. microRNAs in the Regulation of Melanogenesis. *International Journal of Molecular Sciences*. 2021; 22(11):6104. https://doi.org/10.3390/ijms22116104

B.



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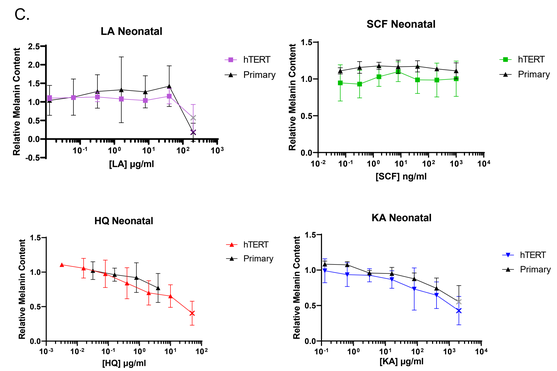

Figure 4. hTERT melanocytes show expected responses to stimulators and inhibitors of melanogenesis. (A) We tested the model with four compounds: the two tyrosinase (TYR) inhibitors hydroquinone (HQ) and kojic acid (KA), and the two stimulators latanoprost (LA) and stem cell factor (SCF). Cells were treated for 4 days and then lysed. (B) We then observed melanin content via A405. (C) We observed a decrease in melanin content for the inhibitor treatments that matched our observations from primary cells. We did not see a response in the stimulator-treated cells; this was also observed in the primary cells, suggesting that this is characteristic of neonatal cells or of the donor source. We observed toxicity at high concentrations of LA, HQ, and KA (x data points in the graph)

Summary

hTERT-immortalized Neonatal Melanocytes:

- Have greatly increased longevity compared to primary melanocytes.
- Maintain primary cell characteristics such as melanin secretion.
- Aid keratinocytes and fibroblasts in forming robust 3D organotypic co-cultures.
- Respond to inhibitor treatment, show muted responses to stimulator treatment.

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