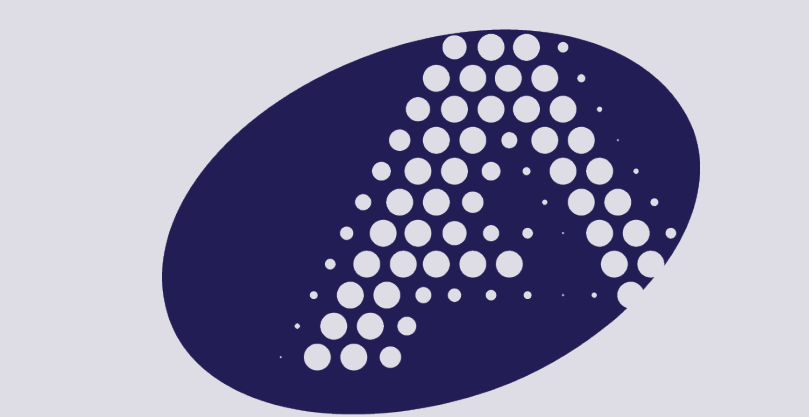


Development of hTERT-Immortalized Neonatal Melanocytes for Toxicity Studies and Melanogenesis Regulation

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Abstract

Melanocytes are the cells that produce melanin, and they are involved in the process of epidermal pigmentation, which involves two main steps. First, melanocytes perform a variety of complex biochemical and physiological steps to produce, package, and exocytose melanin-containing melanosomes. Second, the melanosomes are taken up by neighboring keratinocytes where the stored melanin protects the underlying tissues from damaging UV radiation. In addition to playing an integral role in skin pigmentation, the dysfunction of melanocytes is associated with skin disease and skin cancer. Therefore, it is important to have a reliable model system to study melanocytes and associated processes. Primary cells offer one model system to study pigmentation and dermal agents that may disrupt the melanogenesis; however, they are limited by lifespan and donor-to-donor variability. 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 the ability to create melanosomes in 3-D organotypic co-cultures, the cell line also showed expected levels of responses to several stimulators and inhibitors of melanogenesis. In summary, immortalized melanocytes provide a versatile in vitro cell model for the study of skin toxicology and melanogenesis regulation.

Background

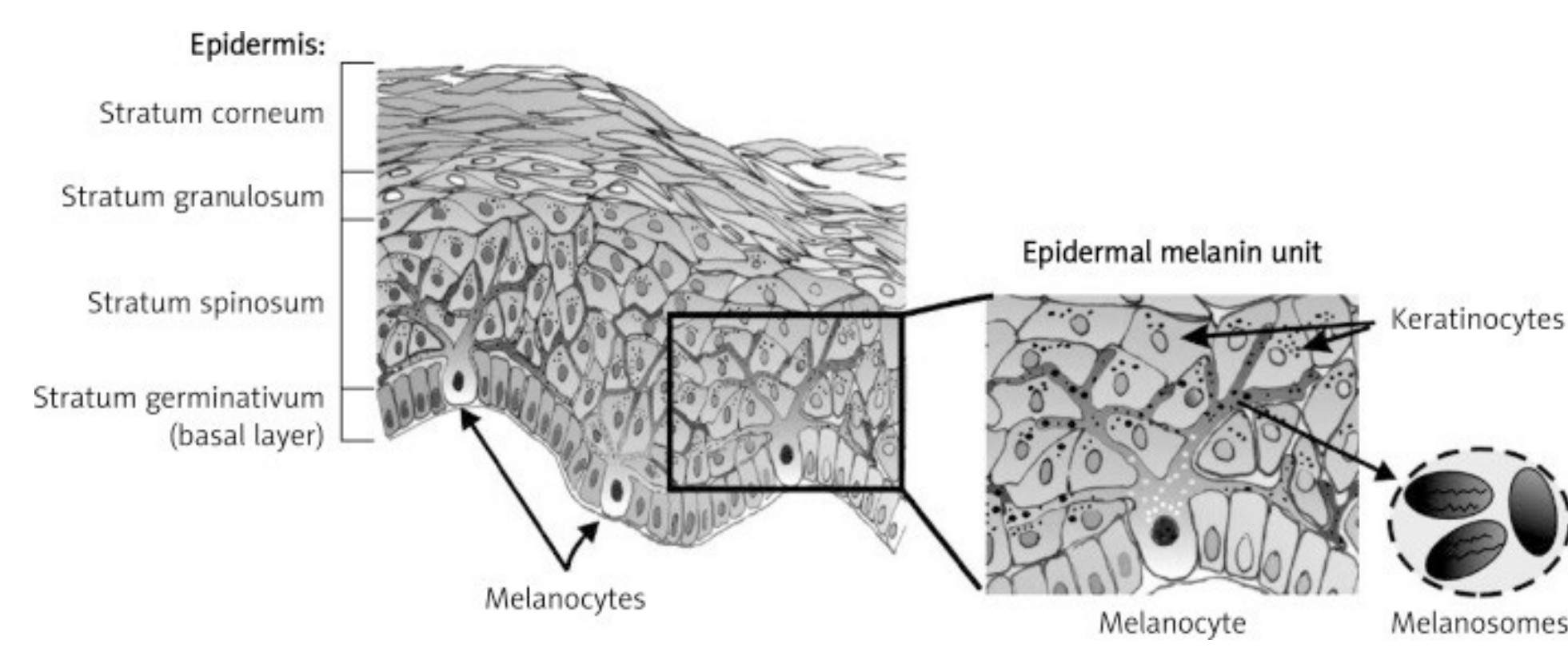


Figure 1. Why Melanocytes? Melanocytes reside in the basal layer of the epidermis and produce protective melanin pigments through a multi-step process. These pigments are packaged into melanosomes, exocytosed, and taken up by neighboring keratinocytes. Models are needed to understand the complex biochemical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

Results

Generation of Immortalized Neonatal Primary Melanocytes

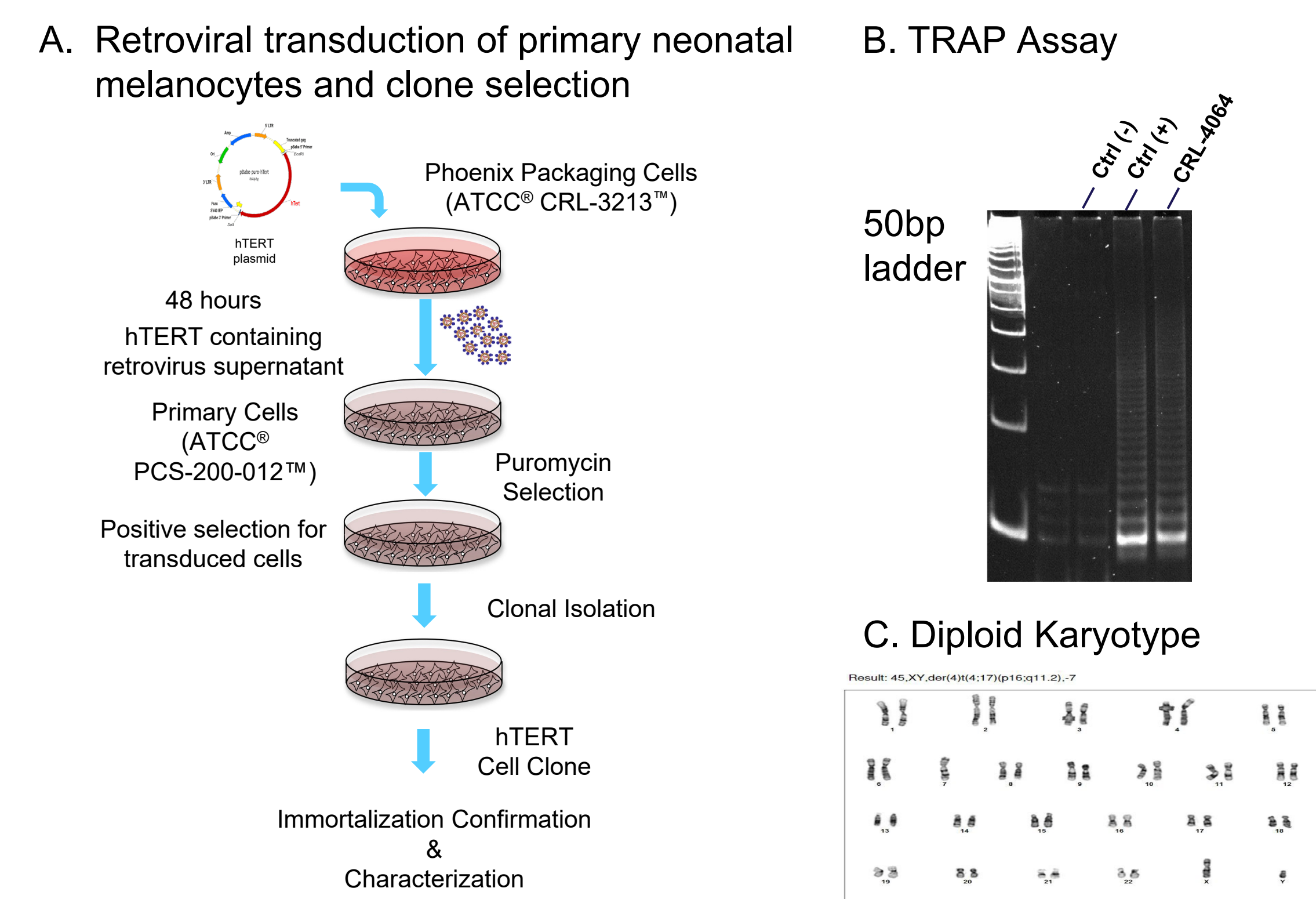


Figure 2. Establishment of hTERT-immortalized neonatal melanocytes. hTERT-immortalized neonatal melanocytes were created by retroviral transduction of the hTERT gene into primary cells. (A) Transduced cells were submitted to puromycin selection and further selected by single cell cloning. (B) High telomerase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

Characterization of Immortalized Neonatal Melanocytes

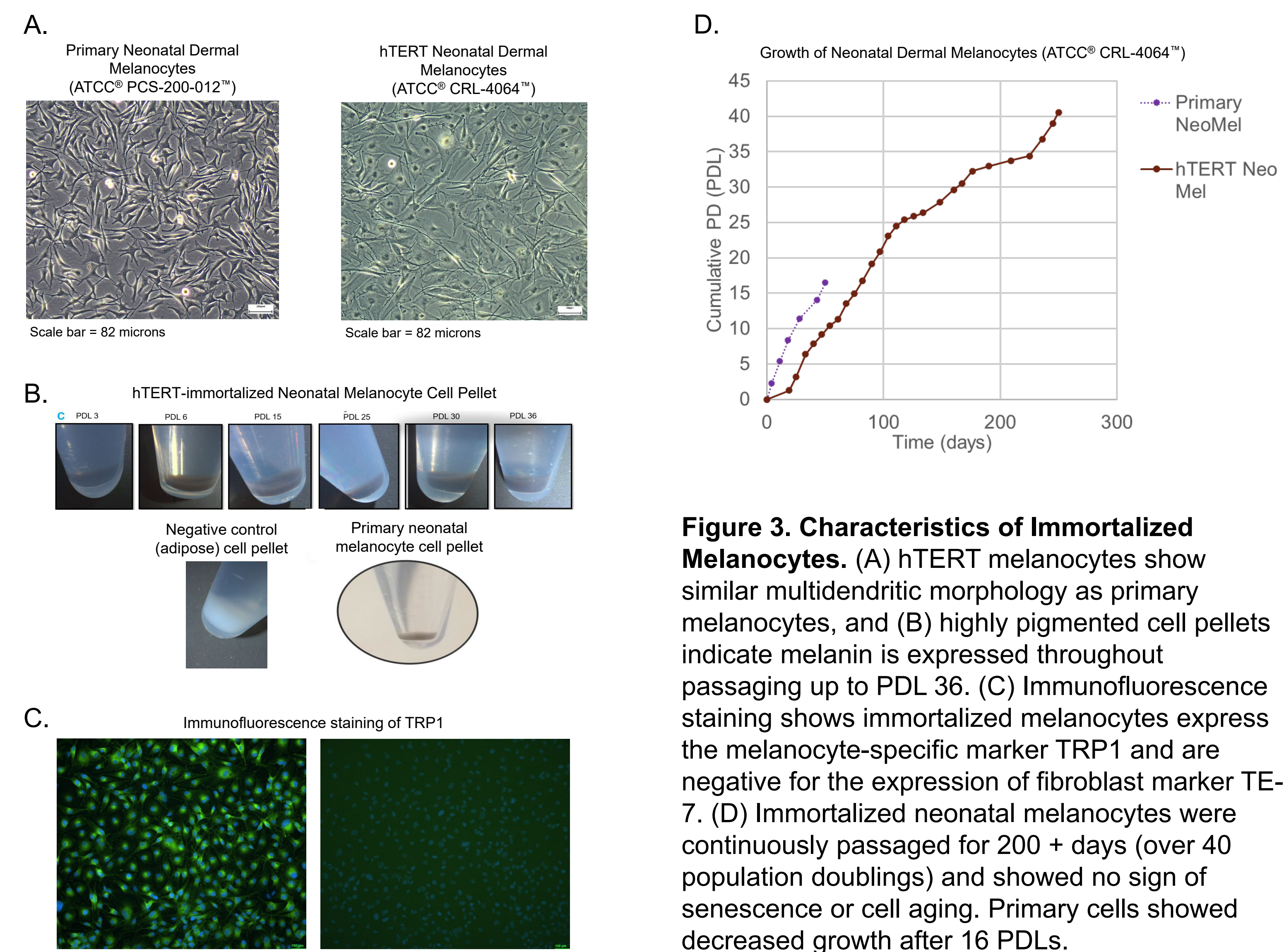


Figure 3. Characteristics of Immortalized Melanocytes. (A) hTERT melanocytes show similar multidendritic morphology as primary melanocytes, and (B) highly pigmented cell pellets indicate melanin is expressed throughout passaging up to PDL 36. (C) Immunofluorescence staining shows immortalized melanocytes express the 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

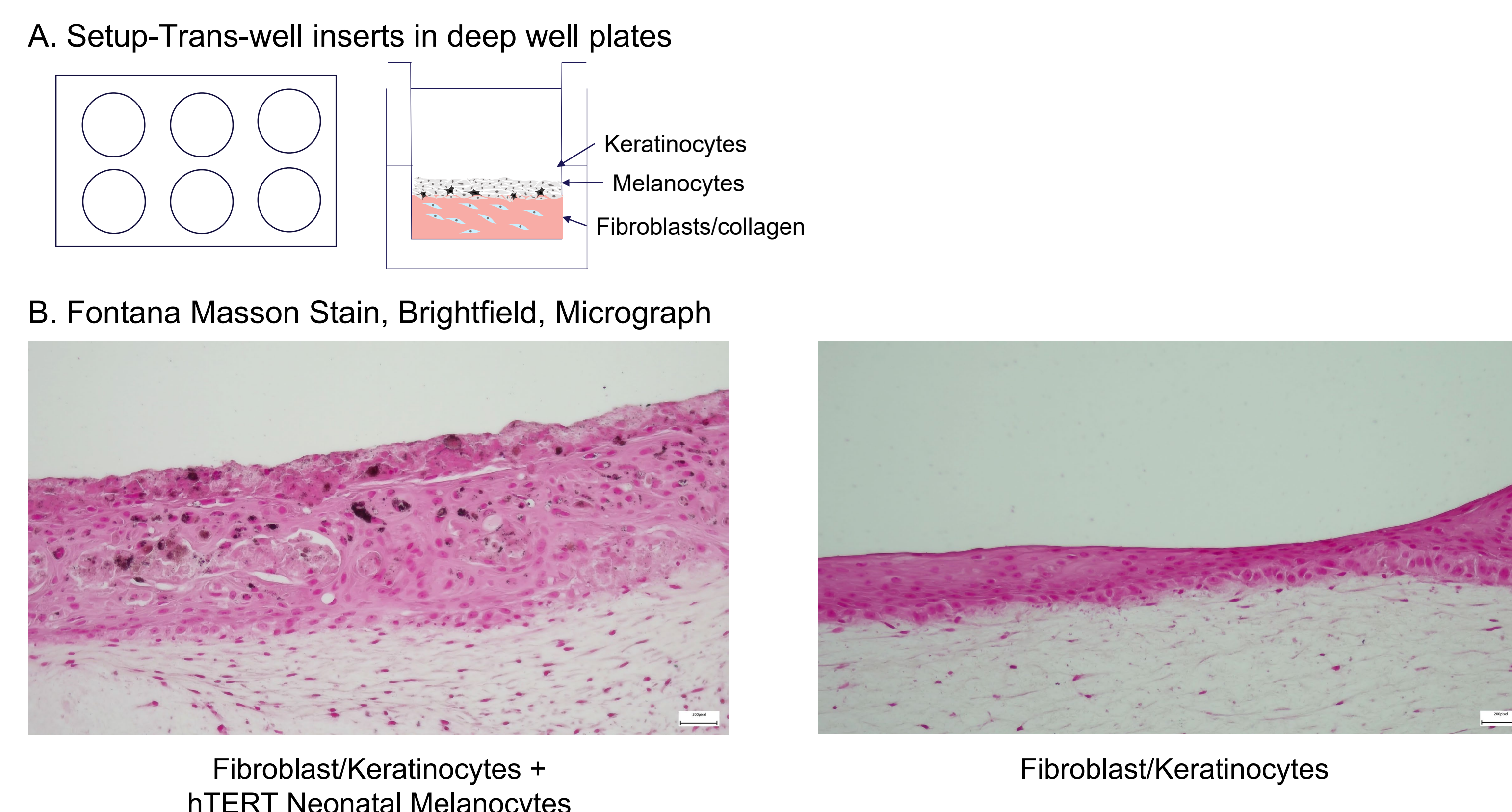


Figure 4. Integration of immortalized melanocytes into 3-D skin co-culture. hTERT-immortalized BJ-5ta (ATCC® CRL-4001™) fibroblasts were embedded into a rat collagen matrix. (A) hTERT-immortalized Ker-CT (ATCC® CRL-4048™) keratinocytes, with or without hTERT melanocytes, were added to the apex of the 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

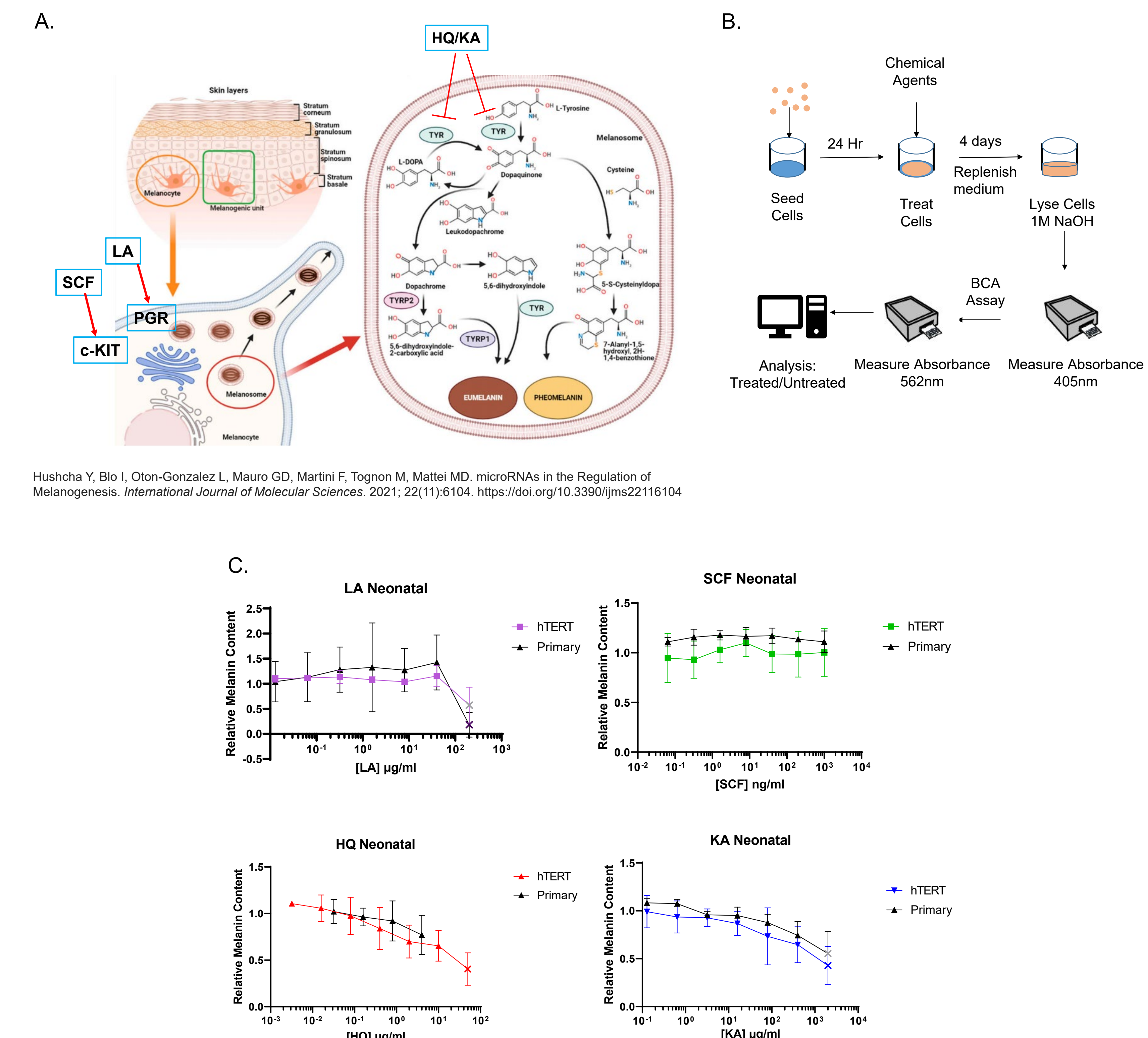


Figure 4. hTERT melanocytes show expected responses to stimulators and inhibitors of melanogenesis. (A) We tested the model with four compounds: the two tyrosinase (TYR) inhibitors hydroquinone (HQ) and kojic acid (KA), and the two stimulators latanoprost (LA) and stem cell factor (SCF). Cells were treated 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 3-D organotypic co-cultures.
- Respond to inhibitor treatment and show muted responses to stimulator treatment.