hTERT-immortalized Adult Dermal Melanocytes as an *In Vitro* Model to Study Melanogenesis

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Abstract

Skin pigmentation is a complex process mediated by melanocytes; mutations in the multiple genes that regulate this process are characteristic of numerous skin disorders, including hyperpigmentation, hypopigmentation, and mixed hyperpigmentation/hypopigmentation. Melanin expression in adult melanocytes is also influenced by additional extrinsic and intrinsic factors such as hormonal changes, inflammation, age, and exposure to UV light. In order to better understand melanocyte biology, there is a need for relevant biological models. The human telomerase reverse transcriptase (hTERT)-immortalized melanocytes described here are a robust model for studying melanocyte function by providing primary melanocyte functionality but exhibiting 'immortalized' characteristics for more than 40 population doublings (PDL) without detectable signs of replicative senescence. These dermal melanocytes maintain consistent expression of the melanocyte-specific marker tyrosinase related protein 1 (TYRP-1) but lack expression of the fibroblast-specific TE7 marker. Melanin production is maintained through the lifecycle, declining only after PDL 45. Functional similarity to primary melanocytes was demonstrated by a concentration-dependent increase in melanin production by melanogenesis enhancers latanoprost and stem cell factor, and a reduction in melanin production in response to antagonist hydroquinone. Last, incorporation of hTERT melanocytes into an organotypic co-culture with fibroblasts and keratinocytes enhances the growth, formation, and pigmentation of the culture. Taken together, these data demonstrate that hTERT melanocytes display a form and function remarkably similar to primary melanocytes, and offer a viable solution for building reliable and complex dermal models.

Results

I. Generation of Immortalized hTERT Melanocytes

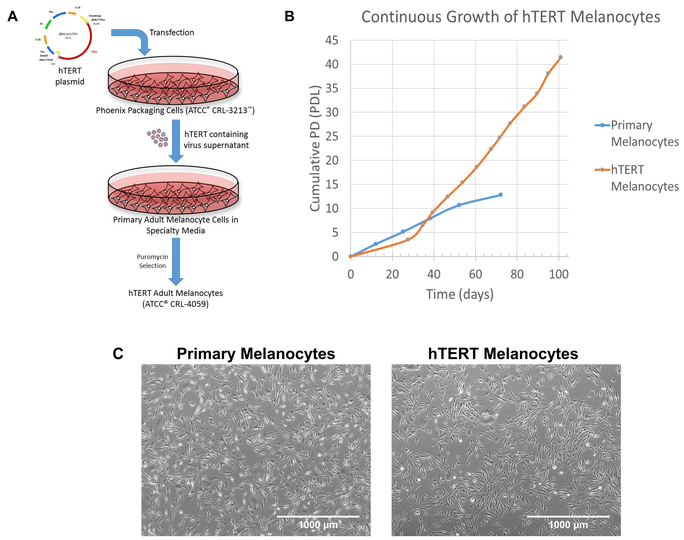

Figure 1. Generation of immortalized primary dermal melanocytes (ATCC® CRL-4059™) via transduction with hTERT. (A) Primary Epidermal Melanocytes (ATCC® PCS-200-013™) were retrovirally transduced with hTERT viral particles in a specialty medium. The stable presence of hTERT was confirmed with a telomeric repeat amplification protocol (TRAP) assay (data not shown). (B) Melanocytes were grown continuously for greater than 40 population doublings (PDL) without signs of replicative senescence. In contrast, primary melanocytes senesced prior to PDL 15. (C) Dermal-derived hTERT melanocytes demonstrate a normal fibroblast-like morphology similar to the primary melanocytes from which they were derived. Scale bar, 1000 µm.

II. Immortalized Melanocyte Cells Maintain Melanin Production

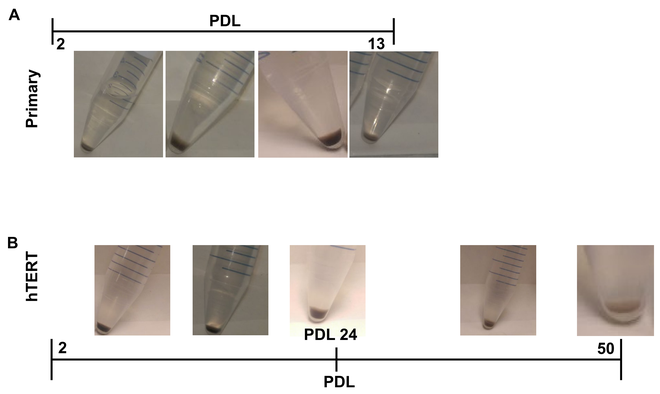

Figure 2. hTERT melanocytes maintain prolonged production of melanin. During continuous culturing of dermal melanocytes, cells were collected and pelleted by centrifugation. The resulting pellets were imaged with a standard digital camera. (A) Primary melanocytes show reduced melanin production by PDL 13 when they senesce. (B) For hTERT melanocytes, high melanin content was maintained until PDL 45 (second image from the right).

III. Marker Expression in hTERT Immortalized Melanocytes

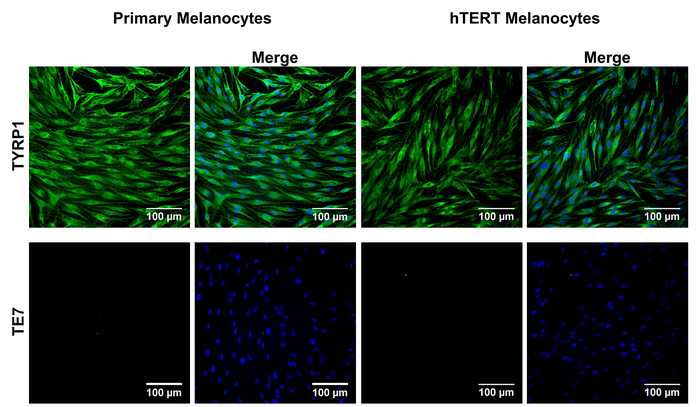

Figure 3. Melanocytes express a melanocyte-specific TYRP1 enzyme, but do not express a fibroblast marker. Primary and hTERT melanocytes were fixed with 4% paraformaldehyde, immunostained with primary antibodies to tyrosinase related protein 1 (TYRP1) and anti-human fibroblast marker (TE7), and then stained with a secondary fluorescent antibody (green). The nuclei were stained with DAPI (blue). Cells were imaged with a fluorescent high-content screening system, and a composite image was generated (merge). hTERT melanocytes show expression of TYRP1 similar to primary melanocytes. Neither of the melanocytes express TE7, a fibroblast specific marker. Scale bar, 100 µm.

IV. hTERT Melanocytes Respond to Stimulators and an Inhibitor of Melanogenesis

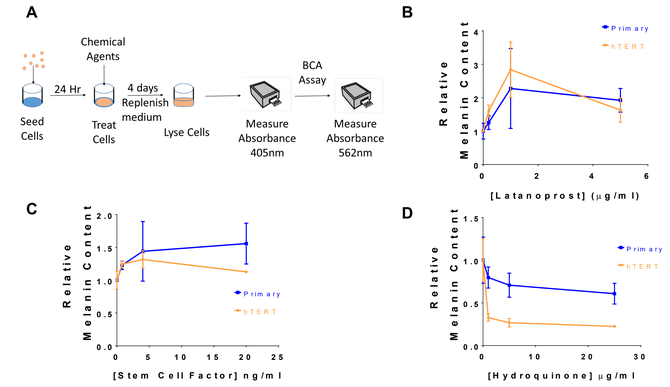

Figure 4. Modulation of melanogenesis with latanoprost, stem cell factor, and hydroquinone. (A) Primary and hTERT melanocytes were seeded into a 96-well plate and then treated with known stimulators (B,C) and an inhibitor (D) of melanogenesis. Cells were then lysed by using 1N NaOH, and melanin content was measured via absorbance at 405 nm. Total protein content was determined by BCA assay from an aliquot of the lysate. Melanin content values were adjusted per microgram of total protein for each sample. The relative melanin content was determined by normalizing to the melanin content of untreated cells for each treatment. Both latanoprost and stem cell factor (known stimulators of melanogenesis) increased melanin content of the cells, while hydroquinone (a known inhibitor of melanogenesis) decreased melanin content in a concentration-dependent manner. Data represents the average ± SD of two independent experiments done in triplicate (N=2).

V. hTERT Melanocytes Pigment a 3D Organotypic Skin Culture

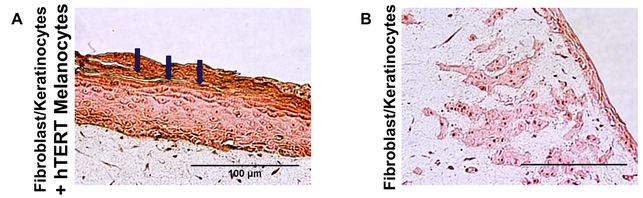

Figure 5. Melanocytes functionally integrate into 3D organotypic skin culture. hTERT-immortalized BJ-5ta (ATCC® CRL-4001™) fibroblasts were embedded into a rat collagen matrix. Next, hTERT-immortalized Ker-CT (ATCC® CRL-4048™) keratinocytes in the presence (A) or absence (B) of hTERT melanocytes were added to the apex of the collagen matrix. After 12 days, cultures were fixed with 4% paraformaldehyde and matrices were embedded in paraffin and sectioned. Sections were stained for melanin (black) with a Fontana-Masson stain kit [Note: melanin deposits (indicated by arrows) should not be mistaken for cell nuclei]. Organotypic cultures lacking hTERT melanocytes failed to develop as robustly as melanocyte-containing cultures, demonstrating the synergistic role of the three cell types. Additionally, skin cultures containing hTERT melanocytes appear overall darker than skin cultures lacking melanocytes, indicating the deposit of melanin into keratinocytes (40x, scale bar = 100 µm).

Summary

- Epidermal-derived melanocytes were successfully immortalized with the catalytic subunit of hTERT.
- hTERT-immortalized melanocytes can grow continuously and maintain the fibroblast morphology of the parental primary cell.
- hTERT and primary melanocytes retain a key marker for melanin biosynthesis, TYRP1, but lack the presence of a fibroblast marker.
- Both primary and hTERT melanocytes respond to stimulators and inhibitors of melanogenesis.
- hTERT melanocytes incorporate into a 3D organotypic skin culture and pigment keratinocytes.
- Taken together, hTERT adult melanocytes are ideal for the study of skin pigmentation.

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