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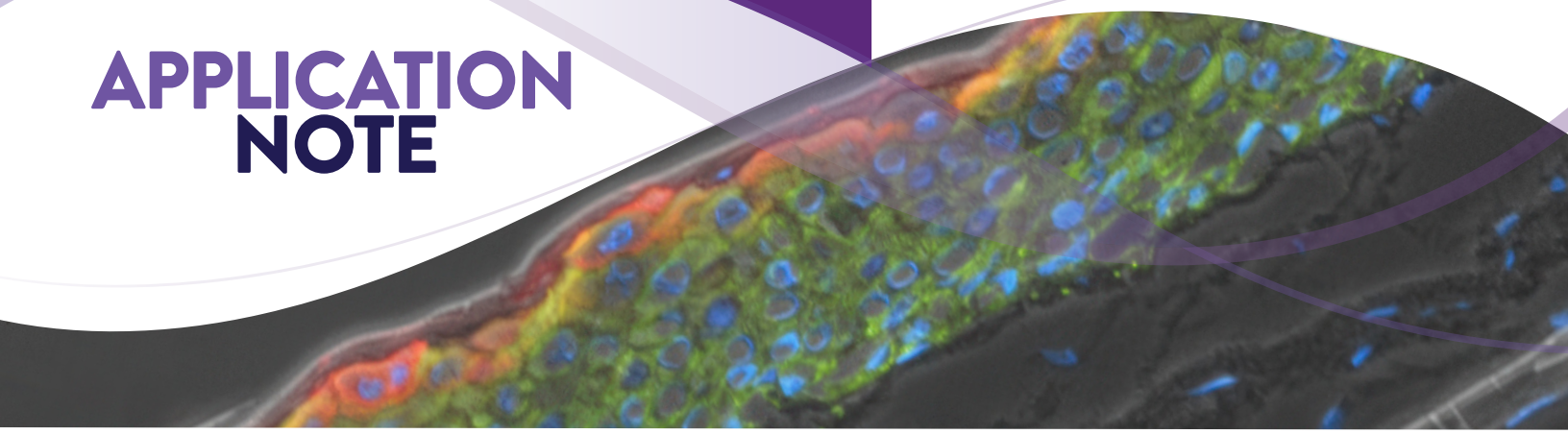
APPLICATION NOTE



IMMORTALIZED HUMAN DERMAL FIBROBLASTS: CONSISTENT, REPRODUCIBLE, AND PHYSIOLOGICALLY RELEVANT MODELS FOR SKIN BIOLOGY STUDIES

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ABSTRACT

While primary human dermal fibroblasts (HDFa) are fundamental to skin biology research, they are limited by donor variability, inconsistent reproducibility, and early cellular senescence. To address these challenges, we developed an hTERT-immortalized HDFa cell line (ATCC® CRL-4066™) using human telomerase reverse transcriptase (hTERT) and mutant cyclin-dependent kinase 4 ($CDK4^{R24C}$). This approach preserves key physiological attributes of the original cells. To further establish its relevance, we validated the utility of the cell line in two representative studies: a drug toxicity study, where Chlorhexidine (CHX) reduced cell viability in a time- and dose-dependent manner, and a coculture experiment with immortalized keratinocytes (ATCC® CRL-4048™) to generate a 3-D in vitro skin model with distinct differentiated layers. These applications highlight the potential of this immortalized HDFa cell line as a robust model for skin biology research.

INTRODUCTION

Human dermal fibroblasts (HDFa) are indispensable for skin biology, playing a critical role in extracellular matrix production, wound healing, and skin regeneration.[1] Because of these functions, HDFa cell lines have long served as gold-standard models for studies on skin disease, skin aging, wound healing, cosmetics development, and toxicology.[2-5] However, primary HDFa have significant limitations, including donor variability, short replicative lifespan, and inconsistent reproducibility across experiments. Lot-to-lot differences further complicate data interpretation, making it challenging to achieve standardized results.

As research continues to move toward more complex in vitro systems, there is a growing need for stable, physiologically relevant HDFa models. Immortalized cells address these challenges by providing a stable, renewable source of fibroblasts that can overcome the restrictions of primary cells and can be used as an alternative model.[6] While the traditional method of immortalizing primary cells through Simian Virus 40 (SV40) can overcome these restrictions, it often leads to the loss of normal cell biofunction.[7] In this study, we aimed to develop an immortalized HDFa cell line using a novel approach that retains the functional characteristics of primary cells and evaluate its suitability a model for applicational studies.

RESULTS WITH MATERIALS AND METHODS

IMMORTALIZATION

Primary HDFa were immortalized using hTERT and CDK4^{R24C} (Figure 1A). Briefly, primary HDFa (ATCC® PCS-201-012™) were thawed in a 37°C water bath for 5 minutes and 5,000 viable cells/cm^2 were seeded to a T-75 flask with complete dermal fibroblast basal medium consisting of Fibroblast Basal Medium (ATCC® PCS-201-030™) and Fibroblast Growth Kit–Low Serum (ATCC® PCS-201-041™). After overnight culture at 37°C with 5% CO_2, the cells were infected with pBABE hTERT retrovirus for 8 hours at a multiplicity of infection (MOI) of 4 in the presence of 4 µg/mL polybrene in 9 mL of complete dermal fibroblast medium. After 8 hours, the hTERT virus medium was aspirated and replaced with fresh complete dermal fibroblast basal medium and cultured for 2 days. After 2 days, the cells were selected using puromycin (Gibco) selection medium (0.5 µg/mL puromycin). The selected cells were then trypsinized and seeded into a T-75 at 5,000 viable cells/cm^2. The next day, the cells were infected with CDK4^{R24C} pLentivirus for 8 hours at a MOI of 4 in the presence of 4 µg/mL polybrene in 9 mL of complete dermal fibroblast basal medium. After 8 hours, the CDK4^{R24C} virus medium was aspirated and replaced with fresh complete dermal fibroblast basal medium and cultured for 2 days at 37°C with 5% CO_2. After 2 days, the cells were selected using puromycin and blasticidin (Gibco) selection medium (0.5 µg/mL puromycin and 2.5 µg/mL blasticidin). The pool cells were sub-cultured and passaged until the cells were immortalized. We then isolated single cell clones using a cell sorter and selected the best six single cell clones for further proliferation and morphology, karyotyping, marker, and differentiation testing. Based on our testing results, one clone was selected for master cell bank and product cell line generation. The generated immortalized cell line (ATCC® CRL-4066™) was cultured for 111 population doublings with no signs of replicative senescence (Figure 1B). The cells also showed fibroblast-like morphology (Figure 2A) similar to that of the primary HDFa and had an apparently normal diploid karyotype (Figure 2B).

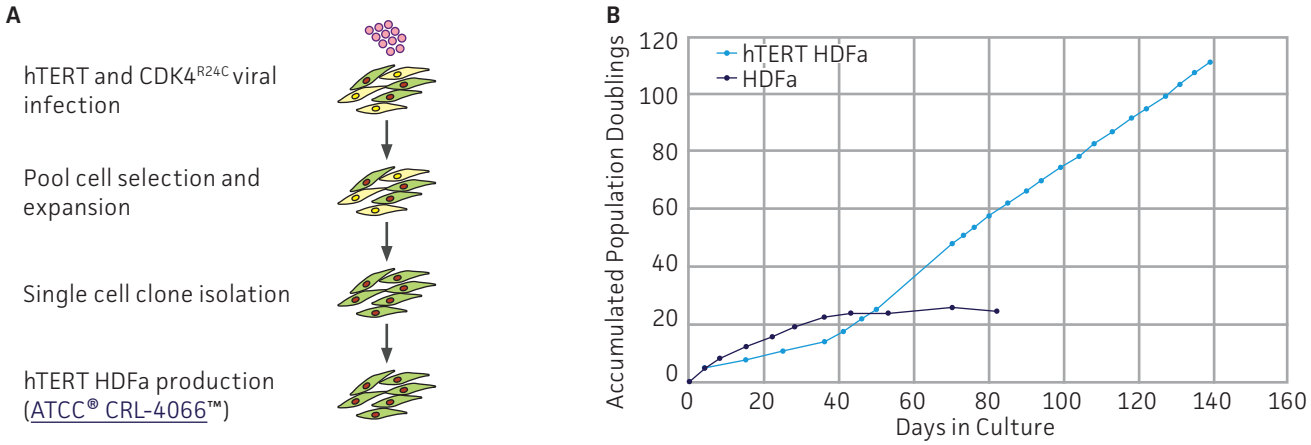

Figure 1: Immortalization of human primary dermal fibroblasts (HDFa). (A) Primary dermal fibroblasts (ATCC® PCS-201-012™), shown as a heterogenous population (yellow and green cells) were infected with hTERT retro virus first and then CDK4^{R24C} lentivirus in the following passage. The hTERT HDFa (ATCC® CRL-4066™) cell line was generated with a single clone shown as a homogenous population of identical cells (green cells). (B) hTERT HDFa cells maintained consistent growth over 111 population doublings while primary HDFa underwent senescence around 26 doublings.

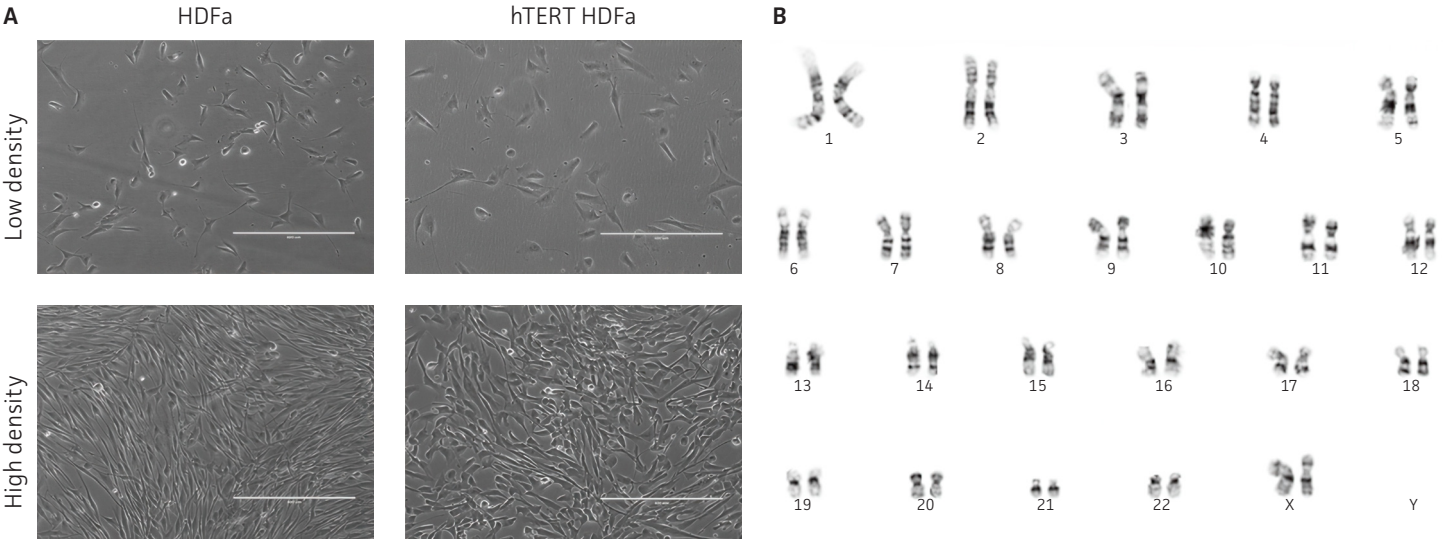

Figure 2: Evaluation of morphology and karyotype. (A) Morphology of primary HDFa and hTERT HDFa at low and high densities. hTERT HDFa showed similar morphology to that of primary cells. (B) hTERT HDFa retained a normal diploid karyotype.

MARKER ANALYSIS

For the marker analysis assay, glass chamber slides were coated with 0.1% gelatin solution (Lifeline Cell Technology) and incubated overnight at 37°C with 5% CO_2. The gelatin solution was removed before seeding the HDFa and hTERT HDFa cells. Both were cultured to at least 90% confluence and then the cells were fixed with 4% paraformaldehyde (PFA) (Thermo Fisher Scientific) for 20 minutes. PFA was rinsed off the chambers by rinsing quickly with Dulbecco's phosphate buffered solution (D-PBS) (ATCC® 30-2200™) three times. Cells were blocked for 30 minutes with blocking solution (D-PBS+ 10% goat serum [Sigma] + 0.2% Triton X-100 [Thermo Fisher Scientific]). The cells were stained with either positive cell marker TE-7 (1 µg/mL) (Invitrogen) or negative cell marker pan-cytokeratin (1 µg/mL) (Pan-CK) (Invitrogen) for 1 hour at room temperature and rinsed three times with D-PBS. Cells were then stained with 4 µg/mL Alexa Fluor 555 goat anti-mouse IgG2a secondary antibody (Invitrogen) for 1 hour in the dark at room temperature. Cells were rinsed with D-PBS three times for 10 minutes each rinse and then stained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) for 5 minutes in the dark at room temperature. DAPI was aspirated from the chambers, and the chamber walls were removed. ProLong glass antifade mounting media (Invitrogen) was added to each chamber and a glass coverslip was placed on top of the slide. Images were taken using a fluorescence microscope. Similar to the primary HDFa, hTERT HDFa showed positive staining for TE-7 and negative staining results for Pan-CK (Figure 3).

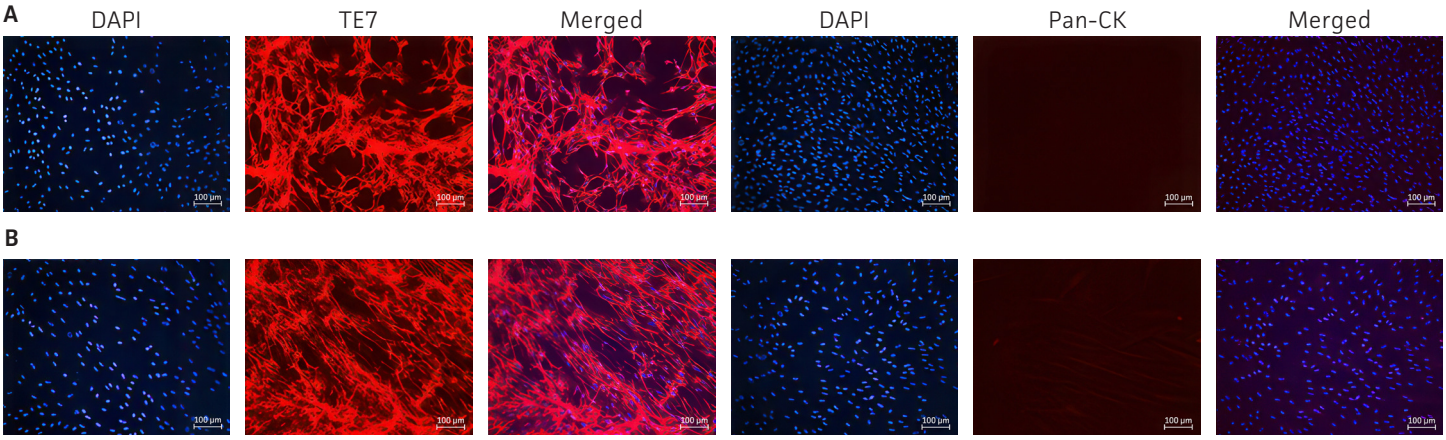

Figure 3: Marker expression in Primary HDFa and hTERT HDFa cells. (A) hTERT HDFa and (B) HDFa cells were grown on glass chamber slides and fixed with 4% PFA. Cells were then stained with TE-7 (positive marker) or Pan-cytokeratin (Pan-CK, negative marker) antibodies (red) and then DAPI (blue). Images were taken using a fluorescence microscope (Nikon).

MYOFIBROBLASTS DIFFERENTIATION

We also tested the hTERT HDFa functional characteristics in comparison to the primary HDFa by conducting a differentiation assay. Glass chamber slides were coated with 0.1% gelatin solution and incubated overnight at 37°C with 5% CO_2. The gelatin coating was removed before seeding the primary HDFa and hTERT HDFa for culture at 37°C with 5% CO_2. After reaching 90-100% confluence, the cells were starved for 1 day in fibroblast basal medium. After starvation, cells were either treated with 20 ng/mL of transforming growth factor beta 1 (TGF-β1) (Sigma) in fibroblast basal medium or just fibroblast basal medium for 3 days to induce dermal fibroblasts to differentiate into myofibroblasts. Cells were then fixed with 4% PFA for 20 minutes and subsequently blocked for 30 minutes with blocking solution (D-PBS + 10% goat serum + 0.2% Triton X-100). After blocking, cells were stained with 1 µg/mL alpha-smooth muscle actin (α-SMA) (Invitrogen) antibody for 2 hours and then stained with 4 µg/mL Alexa Fluor 555 goat anti-mouse IgG2A secondary antibody (Invitrogen) for 1 hour in the dark at room temperature. Cells were rinsed with D-PBS three times and then stained with DAPI for 5 minutes in the dark. DAPI was aspirated from the chambers, and the chamber walls were removed. Mounting media was added to each chamber, and a glass coverslip was placed on top of the slide. Images were taken using a fluorescence microscope. hTERT HDFa retained myofibroblast differentiation ability and showed expression of α-SMA when exposed for 3 days to 20 ng/mL TGFβ1 fibroblast basal media (Figure 4A). When exposed to just fibroblast basal medium for 3 days, both primary HDFa and hTERT HDFa showed no myofibroblast differentiation or α-SMA expression (Figure 4).

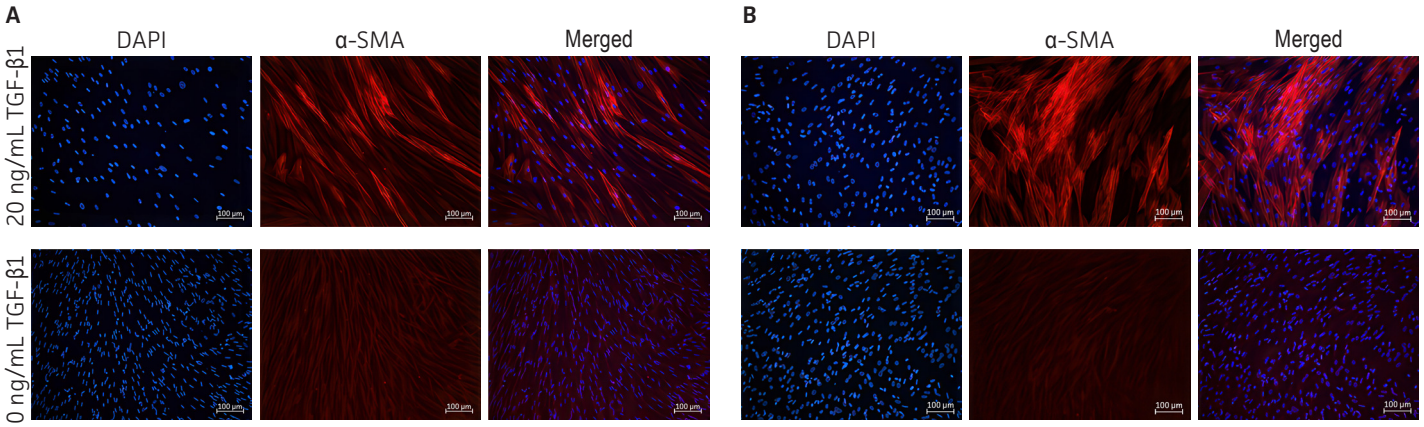

Figure 4: Differentiation of HDFa and hTERT HDFa into myofibroblasts that express alpha-smooth muscle actin. (A) hTERT HDFa and (B) HDFa cells were seeded onto glass chamber slides and cultured to confluence. Cells were starved for 24 hours. Cells were then treated with 20 ng/mL TGFβ1 in fibroblast basal medium for 3 days. After, cells were fixed with 4% PFA and stained with an α-SMA antibody (red) and then DAPI (blue). Cells were only treated with fibroblast basal medium without TGFβ1 as a negative control. Images were taken using a fluorescence microscope (Nikon).

CHX TOXICITY ASSAY

A drug toxicity assay was conducted on hTERT HDFa and primary HDFa to test for a dose- and time-dependent response. Cells were cultured to confluence overnight at 37°C with 5% CO_2 in 96-well plates and then either exposed to 100 µL of 2.5 x 10^{-3}, 5.0 x 10^{-3}, or 1.0 x 10^{-2} % concentrations of chlorhexidine (CHX) in complete fibroblast basal medium for 1, 2, 4, and 6 hours or to 100 µL of 3.125 x 10^{-4}, 6.25 x 10^{-4}, or 1.25 x 10^{-3} % CHX for 3 days. 100 µL CellTiter-Glo (Promega) was added to each well and 200 µL of cell lysate was transferred to an opaque T96 assay plate. Cell viability was determined by measuring luminescence in each well with a GloMax luminometer (Promega) and expressed as a percentage relative to untreated control cells. Cell viability was shown to decrease in a time- and dose-dependent manner in response to CHX (Figure 5).

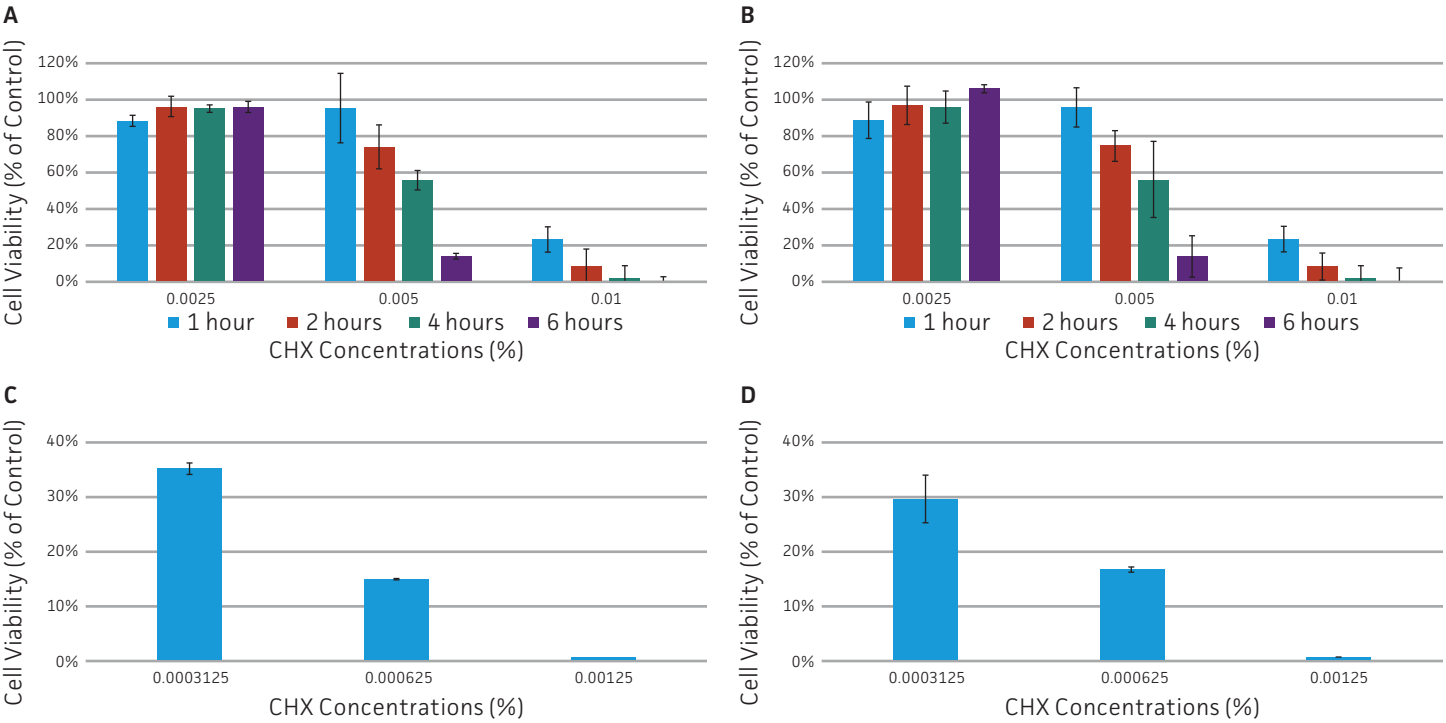

Figure 5: Chlorhexidine (CHX) inhibition effect on cell viability. (A, C) hTERT HDFa and (B, D) HDFa cells were seeded in a 96-well plate and cultured overnight. Three concentrations of CHX media (2.5 x 10^{-3}, 5.0 x 10^{-3}, 1.0 x 10^{-2} %) were added to (A) hTERT HDFa and (B) HDFa cells and treated for 1, 2, 4, and 6 hours. Three concentrations of CHX media (3.125 x 10^{-4}, 6.25 x 10^{-4}, and 1.25 x 10^{-3} %) were added to (C) hTERT HDFa and (D) HDFa cells for 3 days. Cell viability was measured using CellTiter-Glo (Promega Corporation) in a luminometer. The viability of the CHX-treated cells was compared to that of the untreated cells (percent of control).

3-D SKIN MODEL

Generation of 3-D Culture

Immortalized hTERT HDFa (ATCC® CRL-4066™) were cocultured with immortalized keratinocytes (ATCC® CRL-4048™) to create a 3-D in vitro skin model. hTERT HDFa cells were first seeded in a T-75 flask at 5,000 viable cells/cm^2 and cultured until confluent at 37°C with 5% CO_2. Once the cells reached confluence, a 12-well plate with transwell inserts was prepared and coated with the first layer of collagen matrix (Table 1).

Table 1: Composition of the first collagen matrix layer coating

Component	Volume added (µL)	Final concentrations
10X EMEM	297	1X EMEM
200 mM L-glutamine	21.6	1.5 mM L-glutamine
7.5% sodium bicarbonate	252	0.6% sodium bicarbonate
3.1 mg/mL rat tail collagen I	1917	2 mg/mL rat tail collagen I
D-PBS	482.4	N/A
	Total: 2970	

Here, 165 µL of the 2 mg/mL collagen matrix was added to each transwell insert and placed into a 37°C incubator with 5% CO_2 for 1 hour to solidify. After the first collagen coating layer was solidified, the hTERT HDFa cells were trypsinized and 2.5x10^6 viable cells were isolated and resuspended in 1 mL of complete fibroblast basal media. The cells were then combined with the components for the second collagen matrix layer (Table 2).

Table 2: Composition of the second collagen matrix layer

Component	Volume added (µL)	Final concentrations
10X EMEM	429	1X EMEM
200 mM L-glutamine	31.2	1.5 mM L-glutamine
7.5% sodium bicarbonate	364	0.6% sodium bicarbonate
3.1 mg/mL rat tail collagen I	2767.7	2 mg/mL rat tail collagen I
D-PBS	490.1	N/A
2.5x10^6 viable hTERT HDFa cells/mL	208	1.2x10^5 viable hTERT HDFa cells/mL
	Total: 4290	

330 µL of the cells and the collagen matrix mixture was added to each transwell on top the collagen coating layer and then incubated at 37°C with 5% CO_2 for 1 hour to solidify. Complete fibroblast basal medium was added to the apical and basal chambers of each well and transwell insert and cultured for 6 days at 37°C with 5% CO_2. Immortalized keratinocytes (ATCC® CRL-4048™) were seeded to a T-75 flask at 7,000 viable cells/cm^2 and cultured until confluent at 37°C with 5% CO_2 while the 6-day hTERT HDFa collagen matrix was culturing. The keratinocytes were cultured with KBM Gold Basal Medium with KGM Gold SingleQuots supplements (Lonza), excluding GA-1000. After 6 days of hTERT HDFa collagen matrix culture, keratinocytes were trypsinized and 6.0x10^5 viable cells were resuspended in 0.5 mL of KGM Gold Basal Medium and added onto each fibroblast matrix after aspirating complete dermal fibroblast basal medium from all chambers. Complete KBM Gold Basal Medium was added to the basal chambers of each well and cultured for 3 days at 37°C with 5% CO_2. After 3 days, KBM Gold Basal The medium was removed and immediately airlifted, with 3dGRO Skin Differentiation Medium (Sigma) added to only the basal chambers. The collagen matrices were maintained in airlift conditions with 3dGRO medium being changed every 2-3 days for a total of 21 days.

Immunohistochemistry Staining

After 21 days of 3-D culture formation, all medium was removed and the collagen matrices were fixed with 4% PFA for 2 hours at room temperature. Collagen matrices were rinsed once with D-PBS and then shipped to VitroVivo Biotechnology for paraffin embedding and sectioning. Hematoxylin and eosin (H&E) staining was also done by external services for some of the sections. Unstained slides were sequentially rehydrated in 95%, 70%, and 50% ethanol (Sigma) for 5 minutes at each concentration at room temperature. The slides were then rinsed via submerging in molecular-grade water (ATCC® 60-2450™) for 10 minutes at room temperature. The citrate retrieval buffer (Thermo Fisher Scientific) was heated to 94°C in a water bath in a slide rack container before adding slides and doing retrieval for 20 minutes. The slide rack was then transferred to a room temperature container with room temperature molecular grade water to cool down for 10 minutes. Subsequently, the slides were blocked for 30 minutes with blocking solution (D-PBS+ 10% goat serum + 0.2% Triton X-100) and the double staining primary antibodies were prepared (1 µg/mL Rabbit KRT14 IgG with 1 µg/mL mouse Filaggrin IgG1, and 1 µg/mL Rabbit KRT14 IgG with 0.2 µg/mL mouse Vimentin IgG1). 200 µL of double primary antibodies were added to each slide and incubated for 2 hours. The slides were rinsed gently with D-PBS and then the double staining secondary antibodies were prepared (4 µg/mL Alexa Fluor 555 IgG goat anti-rabbit [Invitrogen] with 4 µg/mL Alexa Fluor 488 IgG1 goat anti-mouse [Invitrogen]). 200 µL of the double antibodies were added to each slide and incubated at room temperature for 1 hour in the dark. Slides were then rinsed with D-PBS three times for 10 minutes each rinse. Slides were stained with DAPI in the dark for 5 minutes at room temperature. DAPI was removed, and slides were allowed to completely dry. Mounting media was added to each 3-D skin section and a glass coverslip was placed on top. H&E staining showed a 3-D skin model consisting of a fully differentiated keratinocyte top layer attached to a bottom hTERT HDFa layer (Figure 6A). Upon immunohistochemistry staining, the skin models also showed distinct differentiated and undifferentiated keratinocytes in the top skin layer. The lower keratinocyte layer stained positively only for keratin 14 (CK14), a positive basal keratinocyte marker. The top keratinocyte layer stained positively for filaggrin (FLG), a positive differentiated keratinocyte marker (Figure 6B). The bottom skin layer stained positively for vimentin (VIM), a positive fibroblast marker (Figure 6C).

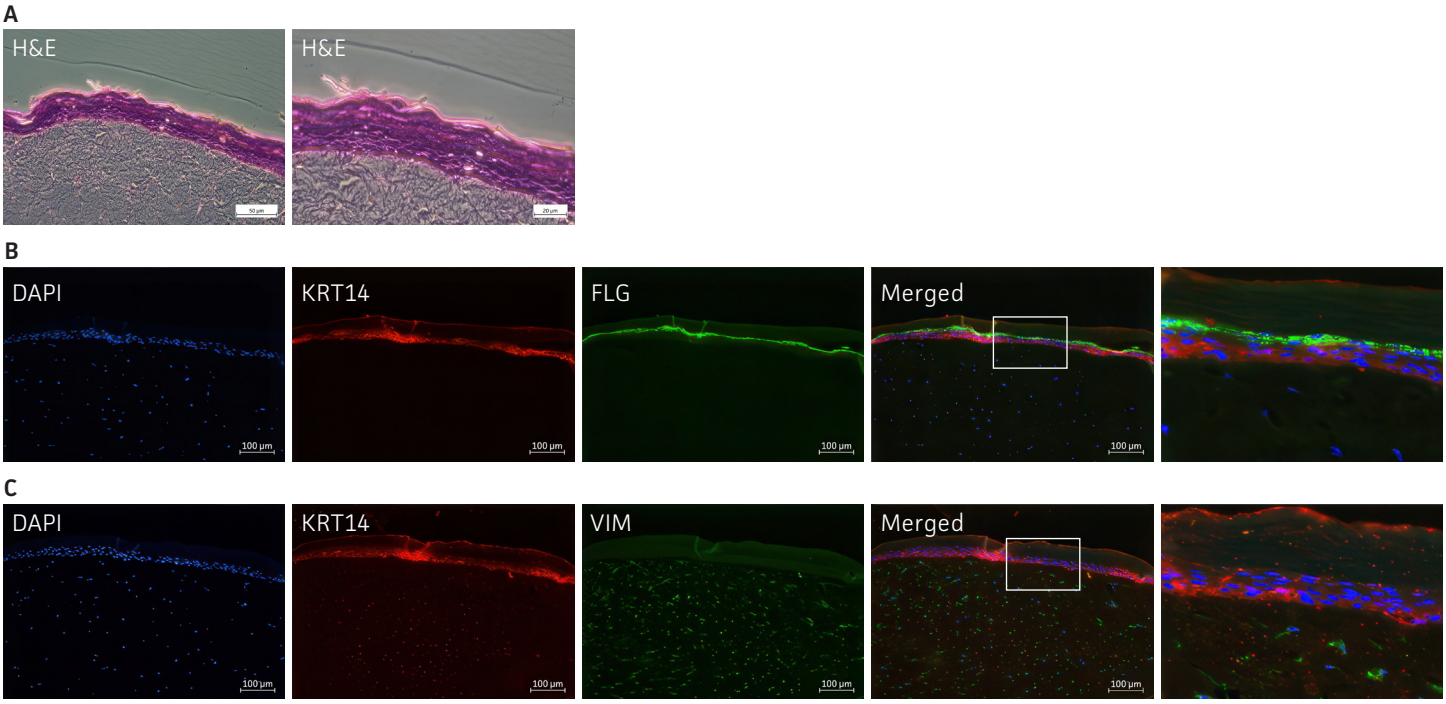

Figure 6: Formation and characterization of a 3-D skin model. Immortalized keratinocytes (ATCC® CRL-4048™) cocultured with immortalized dermal fibroblasts (ATCC® CRL-4066™) for 21 days post air-lift. (A) Representative H&E staining images show a differentiated keratinocyte top layer (dark purple) and a hTERT HDFa collagen bottom layer (light purple). (B) Immunofluorescence staining of 3-D skin model with keratin 14 (KRT14), filaggrin (FLG), and DAPI. (C) Immunofluorescence staining of 3-D skin model with keratin 14 (KRT14), vimentin (VIM), and DAPI.

CONCLUSIONS

Primary dermal fibroblasts have been widely regarded as the standard model for skin biology studies. However, their usefulness is hindered by donor variation, limited lifespan, and reproducibility. In this study, we successfully developed an immortalized primary dermal fibroblast clonal cell line that overcomes these challenges while preserving the same physiological characteristics as the original primary cell line, including TE-7 expression and myofibroblast differentiation capabilities. The immortalized hTERT HDFa cell line demonstrated robust performance in drug toxicity testing, where CHX induced a clear time- and dose-dependent reduction in cell viability, and 3-D skin model generation, where coculture with immortalized keratinocytes produced a stratified tissue structure with distinct basal and differentiated layers. Together, these findings establish the hTERT HDFa cell line as a reliable, physiologically relevant alternative to primary HDFa in skin biology studies. Its stability and functionality make it an ideal model for diverse applications in drug toxicity studies, and advanced in vitro 3-D skin engineering.

REFERENCES

1. Plikus MV, et al. Fibroblasts: origins, definitions, and functions in health and disease. Cell 184(15):3852-3872, 2021. PubMed: 34297930
2. Zorina A, et al. Dermal Fibroblasts as the Main Target for Skin Anti-Age Correction Using a Combination of Regenerative Medicine Methods. Curr Issues Mol Biol. 2023 May 1;45(5):3829-3847. PubMed: 37232716.
3. Stunova A, et al. Dermal fibroblasts-A heterogeneous population with regulatory function in wound healing. Cytokine Growth Factor Rev 39: 137-150, 2018. PubMed: 29395658
4. Groeber F, et al. Skin tissue engineering--in vivo and in vitro applications. Adv Drug Deliv Rev 63(4-5):352-66, 2011. PubMed: 21241756
5. Rahnama M, et al. A comprehensive evaluation of dermal fibroblast therapy in clinical trials for treating skin disorders and cosmetic applications: a scoping review. Stem Cell Res Ther. 2024 Sep 20;15(1):318. PubMed: 39304949
6. Bodnar AG, et al. Extension of Life-Span by Introduction of Telomerase into Normal Human Cells. Science 279(5349): 349-52, 1998. PubMed: 9454332
7. Voloshin N, et al. Practical Use of Immortalized Cells in Medicine: Current Advances and Future Perspectives. Int J Mol Sci. 2023 Aug 12;24(16):12716. PubMed: 37628897

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HDFa-012026-v01