

Abstract

This study will suggest that mesenchymal stem cells (MSC) with their inherent multi-potency are a better alternative to fibroblasts in certain co-culturing situations. Cell-based assays that use primary cells do offer better predictability, but they are hampered by the finite life span of primary cells, which causes inconsistency in the results from donor variation. hTERT-immortalized cell lines solve this problem by offering the functionality of a primary cell with the longevity of a cell line. In this study we characterize the functionality of an hTERT immortalized adipose tissue-derived MSC cell line (hTERT-MSC) and illustrate its use in two co-culturing applications: wound healing and angiogenesis. We confirmed that primary keratinocytes and an hTERT-immortalized keratinocyte cell line, Ker-CT, are able to fully differentiate into skin equivalents in an air-liquid interface (ALI) 3D culture model, when co-cultured with hTERT-MSCs. To confirm the functionality of the co-culture models, both primary keratinocytes and Ker-CT ALI co-cultures were subjected to a scratch assay. Re-epithelialization occurred in both cell lines. Additionally, we established an *in vitro* angiogenesis co-culture model system using TeloHAEC-GFP (an immortalized aortic endothelial reporter cell line stably expressing GFP), and hTERT-MSCs. Results show that the new model can form tubular structures in less than 7 days instead of 14 days compared to co-culture with fibroblasts, and also responds effectively to VEGF stimulation and drug treatments. Furthermore, immunofluorescence staining shows that cells surrounding the tubular structures stain positive for α SMA supporting the physiological relevance of this *in vitro* model system. The co-culture models developed by using hTERT-MSCs in this report provide a more consistent and robust *in vitro* co-culture system for studying wound healing and vascular biology for drug screening and tissue engineering.

Introduction

- hTERT-immortalized cell lines offer the functionality of a primary cell with the longevity of a cell line. hTERT-MSCs are a suitable alternative to primary cells for MSC-based assays.
- Primary keratinocytes can form skin equivalents that mimic the architectural features and behavior of normal skin in a 3D organotypic culture model in an ALI. Ker-CT cultured with hTERT-MSCs comprise a 3D model of the epidermis with high value for investigating drug delivery and toxicity.
- Angiogenesis is a multi-step physiological process; involving endothelial cells and a substrate on which tubules can grow and develop; *in vitro* angiogenesis models provide very useful tool to study angiogenesis; additionally, this model can be used in drug screening applications.

Results

Comparative analysis of cell proliferation, immunosuppressive action, and multi-lineage differentiation of hTERT-MSCs and MSCs from bone marrow, adipose tissue, and umbilical cord blood

CD Marker	CD14 (%)	CD19 (%)	CD34 (%)	CD45 (%)	CD29 (%)	CD44 (%)	CD73 (%)	CD90 (%)	CD105 (%)	CD166 (%)
BM-MSC	0.26	0.07	2.91	0.15	100	99	100	100	100	94
AT-MSC	0.55	0.23	2.86	0.29	100	100	100	99	100	90
UC-MSC	0.52	0.79	1.50	0.47	100	90	95	96	94	95
hTERT-MSC	0.25	0.23	0.99	0.55	100	100	100	100	99	96

Table 1. Flow cytometric analysis of surface marker expression in BM-, AT-, UC-, or hTERT-MSC All cells were negative for surface markers CD14, CD19, CD34, and CD45 and positive for CD29, CD44, CD73, CD90, CD105, and CD166 (Table 1), which meets International Society for Cellular Therapy (ISCT) guidelines.

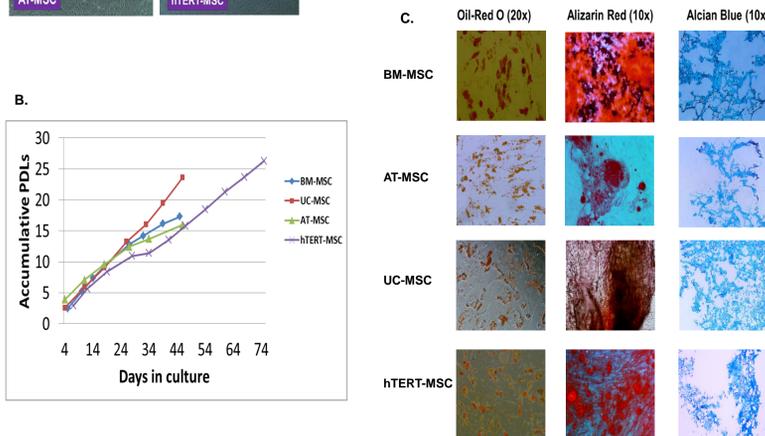


Figure 1. Characterization of MSCs from BM, AT, and UCB and hTERT-MSCs: To compare cell morphology, proliferation rate, and surface marker expression, MSCs were cultured in their corresponding MSC growth media for at least ten passages. A) MSCs exhibited a spindle-shaped morphology and similar growth rates although Umbilical Cord-derived Mesenchymal Stem Cells (UC-MSCs; ATCC® No. PCS-500-010) appeared to have the highest growth rate. B) Bone Marrow-Derived Mesenchymal Stem Cells (BM-MSC, ATCC® No. PCS-500-012), UC-MSCs, and Adipose-Derived Mesenchymal Stem Cells (AT-MSCs; ATCC® No. PCS-500-011) could be expanded for at least 15 population doubling levels (PDLs) post-thaw prior to senescence while hTERT-immortalized Adipose-derived Mesenchymal Stem Cells (ATCC® No. SCRC-4000™) demonstrated the highest proliferative capacity and could be cultured for more than 25 PDLs without any indication of senescence. C) BM-MSCs, AT-MSCs, UC-MSCs, and hTERT-MSCs displayed equivalent differentiation capacity.

hTERT-immortalized and primary stroma-secreting cells directly and indirectly promote primary keratinocytes to form a stratified, 3D, differentiated epidermis

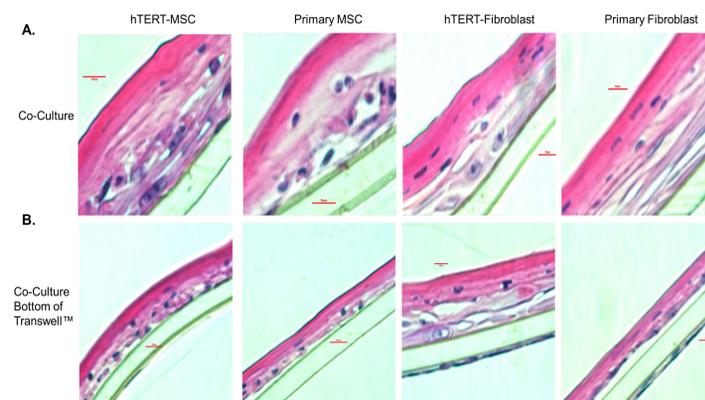


Figure 2. Hematoxylin and Eosin (HE)-stained cross sections of keratinocytes co-cultured with hTERT-immortalized and primary MSCs or fibroblasts differentiated for 21 days. Primary Dermal Fibroblasts (ATCC® No. PCS-201-010), MSCs, or their hTERT-immortalized counterparts were seeded onto A) the Transwell™ (Corning) apical chamber or on B) the underside of the Transwell. The fibroblasts and MSCs were allowed to adhere for 24 hours in Mesenchymal Stem Cell Basal Medium (ATCC® No. PCS-500-030) supplemented with Mesenchymal Stem Cell Growth Kit (ATCC® No. PCS-500-040), BJ-5ta (ATCC® No. CRL-4001™) and primary dermal fibroblasts were grown in Eagle's Minimal Essential Medium (ATCC® No. 30-2003) supplemented with 10% Fetal Bovine Serum (ATCC® No. 30-2020). The keratinocytes were then seeded into the apical chamber and allowed to adhere for 4 hours. Cultures were then maintained as follows: Media was changed to epidermalization medium 1 (EPM1). After 3-5 days media was removed and EPM2 was added to the basal chamber only, with media changes every 2-3 days. The cells remained in culture for 21 days, were cross-sectioned, and then subjected to HE staining. All images were taken at 20 x magnification.

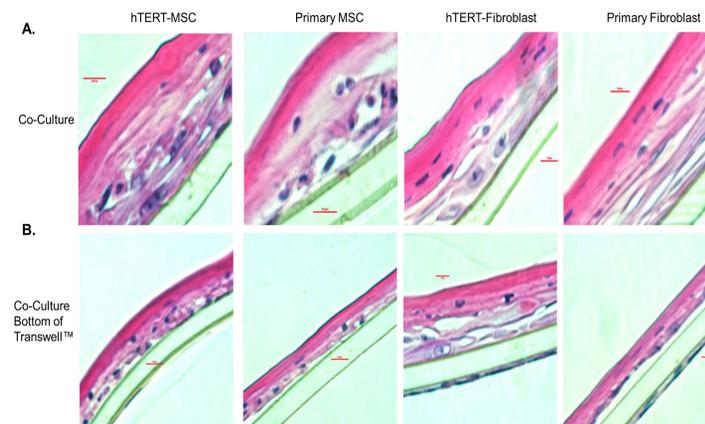


Figure 3. HE-stained cross sections of Ker-CT co-cultured with hTERT and primary MSCs or fibroblasts. Primary fibroblasts, MSCs, or their hTERT-immortalized counterparts were seeded onto A) the Transwell apical chamber or B) the underside of the Transwell, then co-cultured with Ker-CT and processed as in Figure 1.

hTERT-immortalized keratinocytes are able to heal wounds in a scratch assay after 3D differentiation co-cultured with hTERT-MSCs

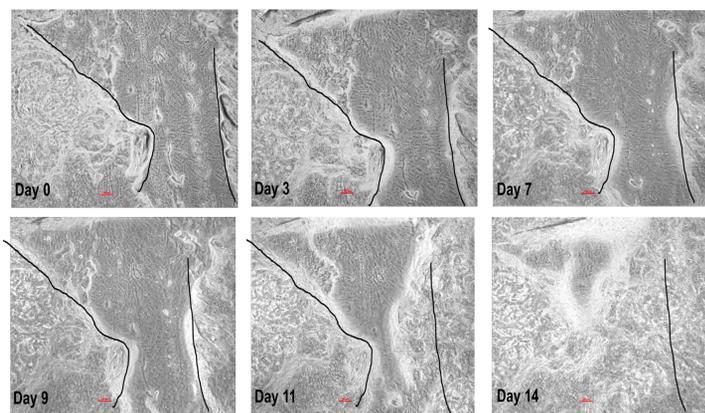


Figure 4. Ker-CT co-cultured with hTERT-MSCs healed after scratch test. Ker-CT cells were seeded onto Transwells containing hTERT-MSCs in the basal chamber as described in figure 1. After 21 days of differentiation, the apical chambers of the Transwells were scratched using a P1000 tip. Media (EPM2) was changed every 2-3 days for a total of 14 days. Phase images at a 10 x magnification were taken at the indicated days to observe the wound healing. Black lines indicate the size of the wound at day 0.

TeloHAEC-GFP and hTERT-MSC co-cultures closely represents *in vivo* angiogenesis

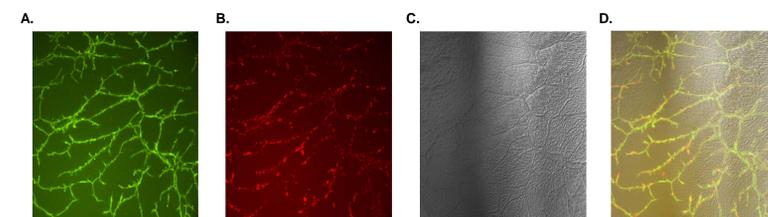


Figure 5. Establishment of TeloHAEC-GFP and hTERT-MSC co-culture angiogenesis. A) TeloHAEC-GFP (ATCC® No. CRL-4054™) co-cultured with hTERT-MSCs for 7 days in the optimized angiogenesis medium displayed a long branching organization and B) exhibited immuno-reactivity to an α SMA antibody (Sigma), which D) co-localized with the TeloHAEC-GFPs. C) Phase contrast microscopy indicated the 3D structure of the tubes.

The TeloHAEC-GFP and hTERT-MSC co-culture model provides a real-time, *in vitro* angiogenesis assay system

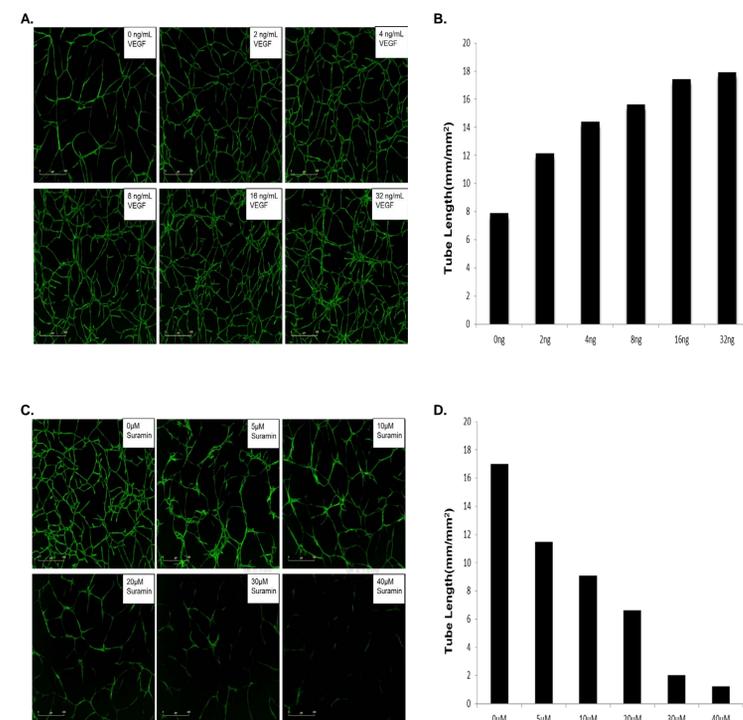


Figure 6. Validation of the TeloHAEC-GFP and hTERT-MSC co-culture angiogenesis assay system. TeloHAEC-GFP cells were co-cultured with hTERT-MSC as in Figure 5 and treated with VEGF or suramin at the indicated concentrations. A) and C) indicate GFP epifluorescence. B) and D) indicate quantitation of the tubule lengths.

Summary

- The hTERT-MSCs exhibit all the characteristics and attributes usually associated with primary MSCs. However, hTERT immortalization also provides the benefit of longevity and expansion capabilities, eliminating concerns with donor variability.
- hTERT-MSCs provides an excellent "companion" cell for the development of 3D cell-based assays.

References

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