A Serum- and Bovine Pituitary Extract (BPE)-Free Medium Supporting Long-Term, Feeder-Free Expansion Of Primary Human Epidermal Keratinocytes that Retain Their Air-Liquid Interface Differentiation Potential

Yaoyao Chen[1], Victoria Stephenson[1], John Stingl[2], Allen C. Eaves[1,2,3], Sharon A. Louis[2], and Wing Chang[1]

[1]STEMCELL Technologies UK Ltd., Cambridge, United Kingdom; [2]STEMCELL Technologies Inc., Vancouver BC, Canada; [3] Terry Fox Laboratory, BC Cancer, Vancouver BC, Canada

INTRODUCTION

Primary human epidermal keratinocytes (HEKs) derived from the skin typically undergo growth arrest at approximately 15 - 30 population doublings (PDs) when maintained in traditional feeder-free culture medium formulations. To circumvent this early senescence, we developed DermaCult™ Keratinocyte Expansion Medium, a serum- and BPE-free medium that promotes long-term growth (> 30 PDs) of HEKs while maintaining their air-liquid interface (ALI) differentiation potential.

METHODS

To measure cell expansion over the entire lifespan of the culture, commercially supplied HEKs derived from either neonatal or adult skin were thawed and seeded onto uncoated tissue cultureware at a density of 1 - 2.5 x 10^3 cells/cm^2. Full-medium changes were performed every other day, and cells were enzymatically dissociated and passaged when cultures reached approximately 50 - 70% confluence. At various time points, keratinocytes were analyzed by qPCR and flow cytometry to detect expression of basal and differentiated cell markers. Keratinocytes were also differentiated by increasing the calcium concentration when the cells were in conventional monolayer culture or in ALI culture. Calcium-induced monolayer differentiation for 3 days was analyzed by immunostaining of basal, suprabasal and tight junction markers. ALI cultures at 14 days were paraffin embedded and sectioned. Cellular architecture was examined by hematoxylin and eosin (H&E) stain and immunostaining for basal and suprabasal markers.

RESULTS

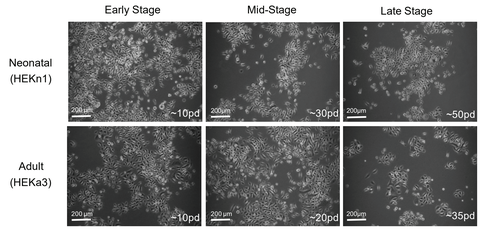


FIGURE 1. Representative Morphology of Primary Human Epidermal Keratinocytes

Representative 10X phase contrast images of HEKs (1 neonatal, 1 adult) cultured long term in DermaCult™. HEKs cultured in DermaCult™ maintain a cobblestone morphology throughout the culture period. The early, mid-, and late stages correspond to the number of population doublings (pd) reached. As adult lines tend to have a reduced longevity compared to neonatal lines, these timepoints were assigned lower population doublings.

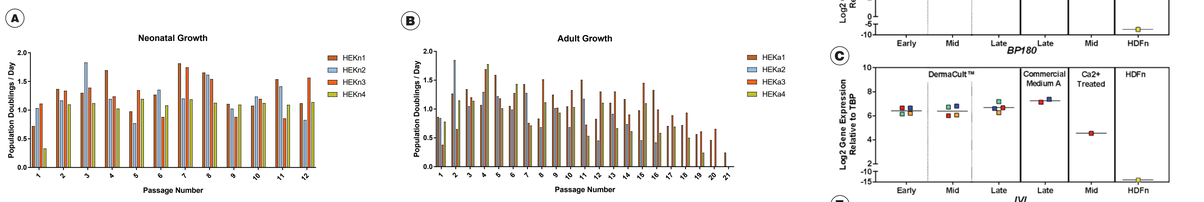


FIGURE 2. Growth Rate of HEKs Maintained in DermaCult™

HEKs cultured in DermaCult™ maintain a good proliferation rate over long term in culture, as observed by the population doublings per day at each passage. **(A)** The neonatal lines were maintained until passage 12, while **(B)** the adult lines were maintained until senescence.

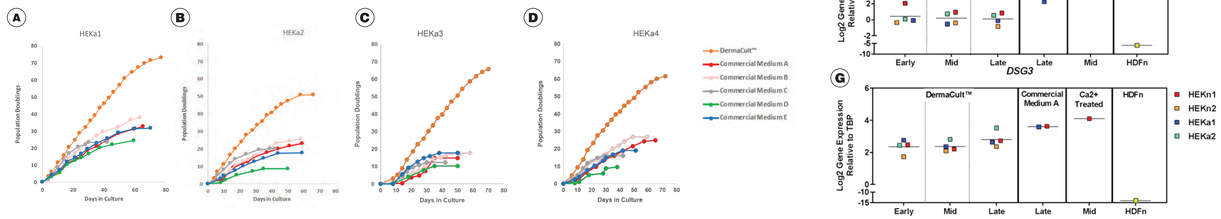


FIGURE 3. Growth Curves of HEKs Maintained in DermaCult™ and Commercially Available Media

Adult HEKs cultured in DermaCult™ show an increased proliferation rate and extended longevity compared to HEKs cultured in other commercial media (n = 4). Growth was assessed until senescence was reached.

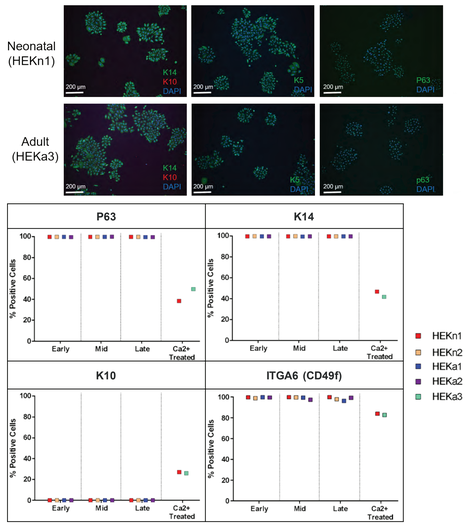


FIGURE 4. Representative Immunofluorescence Images of HEKs Maintained in DermaCult™

ICC of HEKs (1 neonatal, 1 adult) maintained in DermaCult™ showed expression of the basal markers K14, K5, and p63 (green) and absence of the suprabasal marker K10 (red). Widespread co-labelling of the basal markers and the nuclear stain DAPI (blue) was observed. Magnification: 10X.

FIGURE 5. Flow Cytometry Showed the Expected Protein Expression of Key Markers

Flow cytometry analysis was performed for HEKs maintained in DermaCult™ (2 neonatal, 2 adult) at early, mid-, and late stages. Calcium-treated (differentiated) HEKs were included as a control. Over 95% of the cells were positive for the basal markers P63, K14, and ITGA6 (CD49f) and negative for the differentiation marker K10. In the calcium-treated HEKs, there was upregulation of K10 and downregulation of P63, K14, and ITGA6.

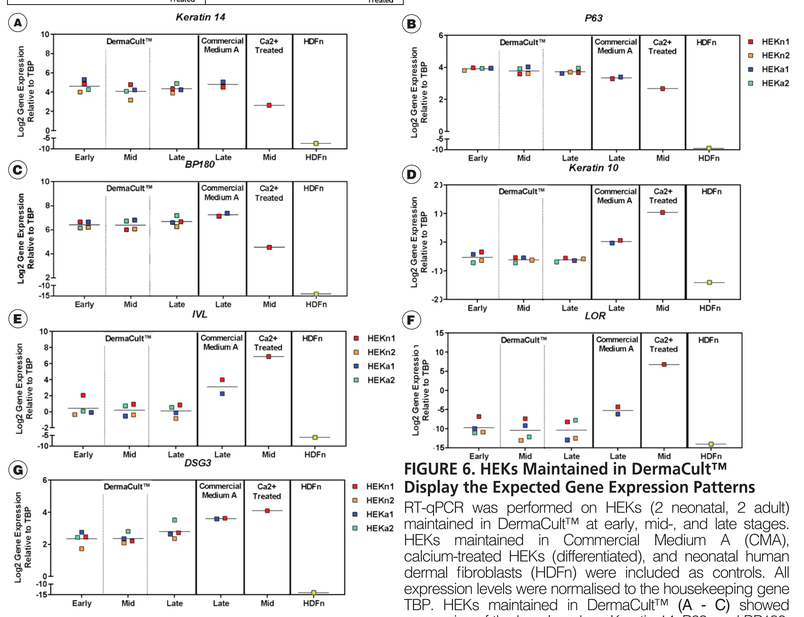


FIGURE 6. HEKs Maintained in DermaCult™ Display the Expected Gene Expression Patterns

RT-qPCR was performed on HEKs (2 neonatal, 2 adult) maintained in DermaCult™ at early, mid-, and late stages. HEKs maintained in Commercial Medium A (CMA), calcium-treated HEKs (differentiated), and neonatal human dermal fibroblasts (HDFn) were included as controls. All expression levels were normalised to the housekeeping gene TBP. HEKs maintained in DermaCult™ **(A - C)** showed expression of the basal markers Keratin 14, P63, and BP180. This was comparable to HEKs in CMA and greater than the calcium-treated HEKs. HEKs maintained in DermaCult™ **(D - G)** also showed low levels of expression of the differentiation markers K10, IVL, LOR, and DSG3. These markers were upregulated in the calcium-treated HEKs.

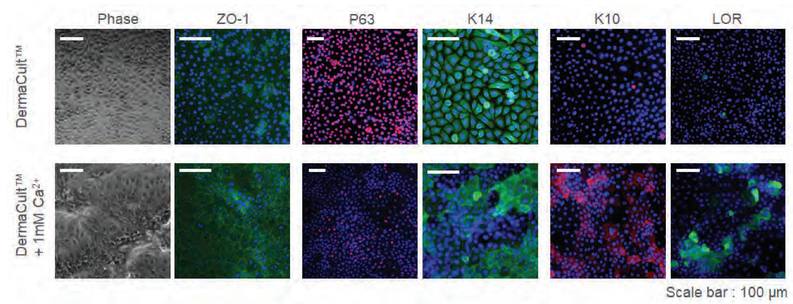
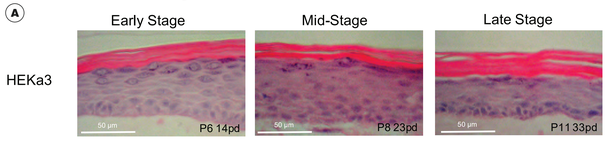


FIGURE 7. High Calcium Induces HEKs Differentiation in DermaCult™

HEKs (adult; HEKa3) were differentiated by increasing the calcium concentration when the cells were in conventional monolayer culture. This resulted in the expression of K10, LOR, and formation of ZO-1 tight junctions, with concomitant downregulation of basal markers P63 and K14.



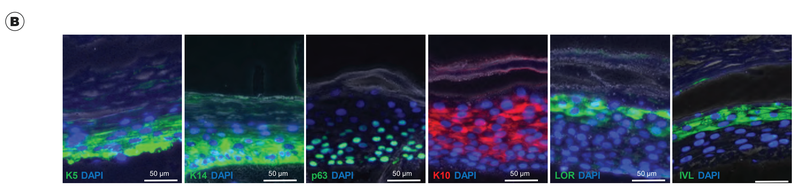
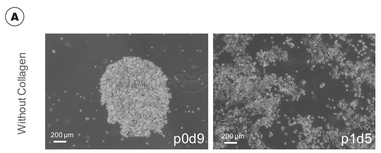


FIGURE 8. HEKs Maintained in DermaCult™ Differentiate at Air-Liquid Interface

(A) HEKs (HEKa3) maintained in DermaCult™ retain their capacity to differentiate at ALI. A representative adult keratinocyte line was able to differentiate at ALI at early, mid-, and late stages. H&E staining shows a multi-layered structure with cuboidal basal cells and a clear cornified layer.
(B) HEKs differentiated at ALI showed the expected marker expression patterns with immunofluorescent stain. K5, K14 and P63 were expressed in the basal layers while K10, LOR and IVL were expressed in suprabasal layers. In these images, fluorescent and phase contrast photos were overlayed. Magnification: 20X.



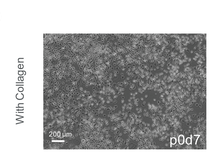


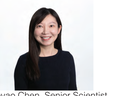
FIGURE 9. DermaCult™ Supports the Derivation of HEKs from Skin Tissue Samples

An adult skin tissue sample was processed using a protocol based on Aasen & Belmonte[1]. Dissociated cells were seeded into DermaCult™ **(A)** with and **(B)** without collagen. Representative images show a typical cobblestone morphology in both conditions. HEKs seeded into DermaCult™ without collagen had a lower attachment but were able to expand and recover upon passaging. Magnification: 4X.

Summary

We have developed an improved BPE-free medium for human primary keratinocyte culture that promotes greater expansion of HEKs and maintenance of their differentiation potential at later passages.

1. Aasen T & Belmonte J. (2010) Isolation and cultivation of human keratinocytes from skin or plucked hair for the generation of induced pluripotent stem cells. Nat Protoc 5: 371–82.



Yaoyao Chen, Senior Scientist
Research & Development
STEMCELL Technologies UK
yaoyao.chen@stemcell.com