Acoustic Cytometric Identification and Initial Characterization Of Limbal Stem Cells Isolated From Human Cornea

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ABSTRACT

Limbal stem cells are known to be precursor cells that repopulate the cornea during wound healing and corneal repair. Limbal stem cells in the basal region give rise to transit amplifying (TA) cells which the migrate to the central cornea. To identify corneal epithelial progenitor populations (stem cells) in adherent cell cultures derived from human limbal tissue, quantitative PCR (qPCR) was used to examine mRNA levels of progenitor markers in Gibco® Human Corneal Epithelial Cells (HCEC) grown in a variety of media systems (both defined and undefined). Minimal differences in transcript levels were observed within the serum-free systems tested, with the exception of SOD2, KRT12, and IVL. Interestingly, when transcript levels of serum-free systems were compared to the SHEM media system: IPO13, TP63, SOD2 were all observed to be at much higher levels (> 2-fold). This may indicate greater retention of progenitor cells cultured under serum-free conditions. HCEC growth rates through seven culture levels (3' – 9') were similar within the serum-free systems, decreasing by the 9' culture level, whereas HCEC grown in the SHEM showed a consistent growth rate (PD/day) during the lifespan examined. Preliminary studies examining dye-excluding (Vybrant® Dyecycle™ Violet) side populations using acoustic flow cytometry are also presented in this work, and suggest the utility of cytometric techniques in quantifying stem cells in expanded limbal cell cultures.

INTRODUCTION

Isolation and culture of limbal cells *ex vivo* have important therapeutic implications, as treatment for limbal stem-cell deficiency (LSCD) requires autologous transplantation of grafts derived from uninjured contra-lateral limbal tissue. Traditional culture systems used to expand HCEC require the use of mitotically-inactive feeder cells (NIH-3T3 or 3T3-J2) and a complex medium, supplementary hormonal epithelial medium (SHEM). This is a time and cost-consuming process that can be significantly streamlined by using a feeder-free culture system. While progenitor marker expression (and clonogenic potential) has routinely been shown to be retained in culture systems containing fibroblast feeders, we present here that a variety of commercially available feeder- and serum-free media systems support robust growth of HCEC and maintenance of progenitor makers (KRT15, KRT19, TP63, ACBG2, SOD2, IPO13) over time.

The p63 gene produces full-length (TAp63) and N-terminally truncated (ΔNp63) transcripts that are alternatively spliced to encode three different p63 isoforms. The p63 gene products are essential for the morphogenesis and the regenerative proliferation of stratified epithelia[1]. In the human corneal epithelium, high levels of ΔNp63α identify limbal stem cells both in vivo and in vitro[2]. Quantification of transcript and protein levels of p63 is therefore important, and identification of stem cells (or TA cells) using a functional assay, like dye-exclusion side-population studies can add significant value to stem cell analysis. Stem cells efflux Vybrant® Dyecycle Violet via an ATP-binding cassette (ABC) membrane pump-dependent mechanism, whereas more differentiated cells retain the probe, allowing visualization of a low-fluorescence "tail" of cells relative to the much larger bulk of Vybrant® Dyecycle Violet-bright cells. The SP phenomenon has been linked to the ABC transporter protein Bcrp1/ABCG2, which effluxes Hoechst 33342 from both rodent and human stem cells[3]. In this work, we present findings on both a limbal progenitor biomarker panel and side population analysis of Gibco® HCEC.

MATERIALS AND METHODS

Gibco® HCEC (Cat. No. C-018-5C) are derived from <30 year old, normal human corneoscleral tissue. The tissue is then trimmed and limbal epithelial cells are selectively released via enzymatic digestion. Isolated cells are expanded and cryopreserved at the end of the secondary culture (2') according to a proprietary protocol . For growth studies, cells were cultured in the indicated media according to product instructions at initial seeding densities of 5,000 cells per cm^2, and sub-cultured at approximately 90% confluence. SHEM growth media was prepared using the following formulation (Table 1)[4].

For real-time studies, cell cultures at the indicated levels were scraped and lysed. HCEC grown in SHEM media were briefly trypsinized (2 min) using TrypLE™ enzyme to remove fibroblasts before lysis. Total RNA was isolated using Invitrogen Purelink® RNA isolation kit (Cat. No. 12183020), and then reverse transcribed using Invitrogen SuperScript® III First-Strand Synthesis SuperMix (Cat. No. 18080-400) to create cDNA libraries. A 7500 Fast Real-Time PCR System was used in conjuction with Taqman® probesets to determine RNA levels with GAPDH transcripts used for normalization and served as positive controls for all samples. TaqMan® probesets were obtained from Applied Biosystems (Table 2).

For side population studies, Gibco® HCEC were grown to the fourth (4') culture level in Keratinocyte-SFM (cat. No. 17005-042) growth media, then harvested and resuspended at $1x10^6$ cells/ml. Cells were incubated with 5 µM Vybrant® DyeCycle™ Violet ± 5 µM Fumitremorgan-C in growth media at 37°C for 60 minutes, washed with cold HBSS + 2% FBS and resuspended in 5 µM Vybrant® DyeCycle™ Violet ± 5 µM Fumitremorgan-C in growth media at 37°C for another 30 minutes. Cells were washed and resuspended in growth media for analysis. An Attune® Acoustic Focusing Cytometer was used to collect ~120,000 events. Vybrant® DyeCycle™ Violet was excited with a 405 nm laser and emission was collected in 450/40 nm and 603/48 nm bandpass filters.

RESULTS

Figure 1. Growth Rates- Population Doublings per Day

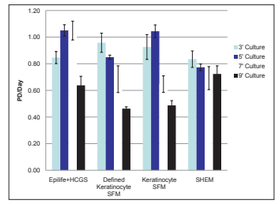

Figure 1- Gibco® HCEC were grown in the media system indicated above to the 9' culture level. At 3', 5', 7', and 9' subcultures, cells were tryposinized and counted using a hemacytometer. Data above are the average of 3 replicate T25 flasks showing average population doublings per day per culture ± standard deviation.

Table 1. SHEM Formulation

Component	Final Concentration	Vendor
DMEM+Glutamax	2/3 of base	Gibco®
F12	1/3 of base	Gibco®
FBS	10%	Gibco®
Human EGF	10ug/ml	Sigma
Hydrocortizone	5mM	Sigma
Bovine Insulin	2.5mg/ml	Sigma
Adenine	24mg/ml	Sigma
Cholera Toxin	16.8ug/ml	Sigma
3,3',5-Triiodo-L-thyronine	3.5mg/ml	Sigma
Antibiotic/ Antimycotic	1X	Gibco®

Table 1- Formulation of SHEM media used in the current study. Media was used for < 30 days after supplementation. When cells were cultured in SHEM, mitomcycin C-treated NIH 3T3 feeder cells were seeded at $5x10^4$ cells/cm^2 and allowed to grow 24h prior to HCEC plating.

Figure 2. Real-time qPCR data- ΔCt from GAPDH Control

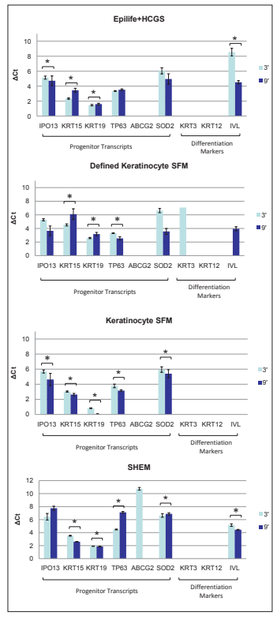

Figure 2- Comparison of progenitor and differentiation marker transcript expression in HCEC cultured under different media conditions. Statistical differences from 3' to 9' are indicated above with asterisks. All Ct values normalized to respective media GAPDH control levels. Data above are the average of 6 replicates ± standard deviation. Asterisks indicate p values of < .0001 (student's t-test), and were considered significant.

Table 2. Applied Biosystem TaqMan® Probe Array

Target	Probe Cat .No.
Cytokeratin 3 (KRT3)	Hs00365080_m1
Cytokeratin 12 (KRT12)	Hs00165015_m1
Cytokeratin 19 (KRT19)	Hs00267035_m1
Involucrin (IVL)	Hs00761767_s1
ΔN-p63α/β/γ (TP63)	Hs00978339_m1
ABCG2 transporter (ABCG2)	Hs1053790_m1
Superoxide dismutase (SOD2)	Hs00167309_m1
Importin 13 (IPO13)	Hs00206301_m1
GAPDH	4333764_0912033

Figure 3. Real-time qPCR data- Fold Change ($2^{-\Delta\Delta Ct}$) from SHEM medium

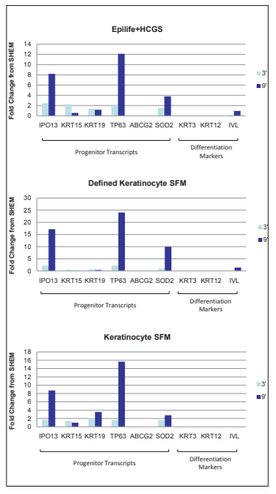

Figure 3- Gibco® HCEC progenitor marker expression higher in serum-free systems. Transcript levels were evaluated in 3' and 9' culture levels in a single donor. Data presented is fold change ($2^{-(\Delta\Delta Ct)}$) from SHEM medium (average of 6 replicates). A value of 1 indicates equivalence as compared to SHEM conditions. Progenitor markers: Importin 13, TP63, and SOD2 showed > 2 fold increase in Keratinocyte SFM (defined and undefined), Epilife+HCGS, and competitor SFM.

Figure 4. Cytometric Analysis of Dye-excluding Side Population (SP) cells

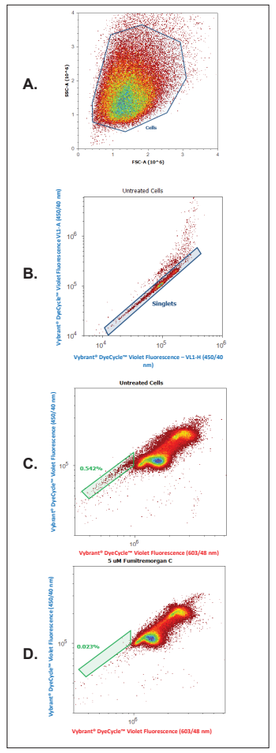

Figure 4- SP Quantification. Gibco® HCEC were grown to the culture level indicated above in Keratinocyte-SFM. Cells were then harvested and incubated with Vybrant® DyeCycle™ Violet stain to identify stem cell side populations. **A:** Representative forward and side scatter dot plot of untreated cells. **B:** A density plot of Vybrant® DyeCycle™ Violet area vs. height was used to eliminate aggregates from analysis. **C:** Untreated cells have a side population of putative stem cells (0.542% of cells) with decreased Vybrant® DyeCycle™ Violet fluorescence due to ABCG2-mediated dye efflux. **D:** The side population appears greatly reduced (0.023% of cells) when the ABCG2 membrane pump is inhibited with 5 µM Fumitremorgan-C. Superior cell focusing provided by the Attune™ Acoustic Focusing Cytometer enabled detection of a very small percentage of putative stem cells.

Figure 5. Morphology

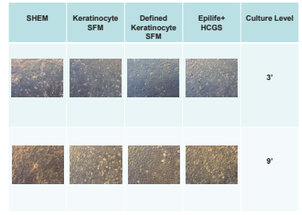

Figure 3- Comparative morphology of Gibco® HCEC expanded under different growth conditions at the 3' and 9' culture levels. Phase-contrast images were acquired at 100X using a Leica DM microscope.

CONCLUSIONS

Minimal differences in qPCR threshold cycle (Ct) values were observed within the serum-free systems tested over 3' to 9' cultures, with the exception of IVL (Figure 2). Interestingly, when transcript levels of serum-free systems were compared to the SHEM media system: IPO13, TP63, SOD2 were all observed to be at > 2-fold higher levels (Figure 3). This may indicate greater retention of progenitor cells cultured under serum-free conditions. HCEC growth rates through seven culture levels (3' – 9') were similar within the serum-free systems, decreasing by the 9' culture level (Figure 1), whereas HCEC grown in the SHEM media showed a consistent growth rate (PD/day) and morphology (Figure 5) during the lifespan examined.

Identifying holoclones visually is a challenge, thus a need for p63 quantification via immunocytochemcial techniques or specific dye labeling are essential. Attune® Acoustic Focusing Cytometer data identified a distinct side population (SP) excluding Vibrant® DyeCycle™ Violet in GIbco® HCEC, indicating the presence of slow-cycling cells in these cultures that reduced in population percentage (0.542% to 0.023%), with Fumitromorgan-C- an inhibitor of the ABCG2 pump (Figure 4). Taken together, this data suggests that qPCR and SP analysis is a novel way to quantify characteristics of stem cell populations in limbal epithelial cells.

REFERENCES

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TRADEMARKS/LICENSING

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