A human corneal epithelial cell line model for limbal stem cell biology and limbal immunobiology.

Running title: Immunobiology of a corneal cell line

Bakiah Shaharuddin[1,2] Sajjad Ahmad[3,4] Nani Md. Latar[1,6] Simi Ali[5] Annette Meeson[1]

[1]Institute of Genetic Medicine, Newcastle University, Newcastle Upon-Tyne, U.K

[2]Advanced Medical and Dental Institute, Universiti Sains Malaysia, Pulau Pinang, Malaysia

[3]St Paul's Eye Unit, Royal Liverpool University Hospital, Prescot Street, Liverpool, UK.

[4]Department of Eye and Vision Sciences, Institute of Ageing and Chronic Disease, University of Liverpool, Liverpool, UK.

[5]Institute of Cellular Medicine, Newcastle University, Newcastle Upon-Tyne, UK.

[6]Department of Surgery, Faculty of Medicine, Universiti Kebangsaan, Malaysia Medical Centre, Kuala Lumpur, Malaysia.

Authors' contribution:

1. Bakiah Shaharuddin: Conception and design, Collection and/or assembly of data, Manuscript writing
2. Sajjad Ahmad: Conception and design
3. Nani Md. Latar: Collection and assembly of data
4. Simi Ali: Conception and design
5. Annette Meeson: Conception and design, Collection and/or assembly of data, Manuscript writing, Final approval of manuscript

Corresponding author: Dr Annette Meeson, PhD, Institute of Genetic Medicine, Newcastle University, International Centre for Life, Newcastle Upon-Tyne, NE1 3BZ, United Kingdom.

Email: annette.meeson@ncl.ac.uk

Tel: +44 (0) 1912418856

FAX: +44 (0) 1912418666

ABSTRACT

Limbal stem cell deficiency is a visually debilitating condition caused by abnormal maintenance of limbal stem cells (LSCs). It is treated by transplantation of donor derived limbal epithelial cells (LEC); the success of which is dependent on the presence and quality of LSC within the transplant. Understanding the immunobiological responses of these cells within the transplants could improve cell engraftment and survival. However, human corneal rings used as a source of LSC are not always readily available for research purposes. As an alternative, we hypothesized that a human telomerase-immortalised corneal epithelial cell (HTCEC) line could be used as a model for studying LSC immunobiology. HTCEC constitutively expressed HLA Class I but not Class II molecules. However, when stimulated by interferon (IFN)-γ, HTCEC then expressed HLA Class II antigens. Some HTCEC cells were also migratory in response to CXCL12 and expressed stem cell markers, Nanog, Oct4 and Sox2. In addition as both HTCEC and LEC contain side population (SP) cells, which are an enriched LSC population, we used these SP cells to show that some HTCEC SP co-expressed ABCG2 and ABCB5. HTCEC SP and NSP also expressed CXCR4 but the SP expressed higher levels, and both were capable of colony formation but the NSP colonies where smaller and contained fewer cells. In addition HTCEC expressed ΔNp63α. These results suggest the HTCEC line is a useful model for further understanding of LSC biology using an *in vitro* approach without reliance on a supply of human tissue.

Key words: limbal stem cell, immunobiology, side population, ABCB5, CXCR4,

INTRODUCTION

Limbal stem cell deficiency (LSCD) is a debilitating eye condition in which, following damage to the corneal epithelium, the cornea fails to regenerate. This failure is due to the loss of limbal stem cells (LSC) and ultimately leads to chronic ocular pain and loss of visions. Tissue availability, small cell yields and patient to patient sample variability can limit limbal studies. Therefore we proposed the use of a human telomerase-immortalised corneal epithelial cell (HTCEC) line as a model for studying LSC biology. The HTCEC was originally derived by forced express of hTERT in human epithelial cells and importantly has been shown to have similarities in stratification and differentiation potential to normal human epithelial cells *in vitro* [1]. We examined HTCEC for stem cell properties, biological and immunological functions. In addition, we further characterised the HTCEC side population (SP). Limbal SP cells (LSP) have features consistent with stem cells [2,3].

MATERIALS AND METHODS

Human tissue use was conducted with ethical approval from the Ethics Committee, Newcastle University, UK and in accordance with the ethical principles of the Declaration of Helsinki.

Limbal Epithelial Cell Culture and Side Population assay.

Both were isolated as described previously [3]. In brief LEC were isolated from tissue using serial trypsinisation, then plated onto irradiated 3T3 fibroblasts and harvested for analysis at day 10. Both LEC and HTCEC were stained with 3µg/ml of Hoechst 33343 dye for 45 minutes prior to FACS analysis.

Human telomerase-immortalised cornea epithelial cell line (HTCEC)

HTCEC was a gift from Professor Kao, University of Cincinnati, USA, and originally derived by Professor Jester, University of California, Irvine, USA. HTCEC were propagated as described previously [4].

Immunocytochemistry (ICC). This was performed as described previously [4, 5]. However briefly, cells for ICC were fixed with cold methanol, washed, permeabilised and non-specific binding sites were blocked by incubation in appropriate blocking serum for 30 minutes. Cells were then incubated with primary antibodies followed by appropriate fluorophore conjugated secondary antibodies. Details of antibodies used are provided in supplementary Table 1.

Semi quantitative Polymerase Chain Reactions (sq-PCR). Was performed as described previously [4, 5]. Oligonucleotide primers and amplification conditions are presented in supplementary Table 2 and [11].

Fluorescence Activated Cell Sorting (FACS). $2x10^5$ cells in 100 µl cells suspensions, for direct immunofluorescence, were stained with 5µl primary antibody for one hour. Cells were then washed and re-suspended in 200 µl buffer solution and analysed using FACS. For indirect immunofluorescence $2x10^5$ were stained with 5 µl primary antibody in 100 µl cells suspensions for one hour, cells were then washed and incubated in appropriate fluorophore conjugated secondary 1:25 dilution for 30 minutes, washed again and resuspended in 200 µl buffer solution and analysed using a FACS Canto and FACS Diva software [BD Biosciences, Oxford, UK]. Antibodies used for FACS analysis are provided in Supplementary Table 1.

Transwell Migration Analysis. $1x10^5$ HTCEC were resuspended in 300 µl defined keratinocyte serum-free medium [Life Technologies, Paisley, UK] and added to the upper chamber of a 5 µm pore diameter 24-well format transwell chamber. The lower chamber contained 200 µl media without cells but supplemented with CXCL12 at 300ng/ml (control was 0 ng/ml) and cells were cultured for 5 hours under standard TC conditions. After this the filters were removed and stained with haematoxylin. Migrant cells were counted (5 randomly selected high power fields/well at x20 magnification).

Microscopy and Imaging. All images were taken using a Nikon Digital Sight-DSFi1 camera and Nikon NIS-Elements D software [Nikon Metrology UK Ltd., Derby, UK] and collated using Adobe Photoshop [Adobe Systems, San Jose, USA]. For fluorescence images an Axioplan F was used and images processed using Axio-Vision40 software [Zeiss, Cambridge, UK].

HLA-typing and HLA expression in HTCEC

HLA-typing was outsourced to NHS Blood and Tissue Bank (Newcastle) courtesy of Dr Carter. To examine HLA expression in HTCEC, cells in culture were treated with IFN-γ1b [Miltenyi Biotec, Bisley, UK] alone, recombinant TNF-α [R&D Systems] alone or with a combination of both, at a pre-optimised concentration of 10ng/ml for 3 days. Samples were then prepared for FACS analysis as described previously [4, 5]. For HLA expression in unstimulated HTCEC, negative control was unstained cells. While for stimulated cells, negative control was unstimulated cells.

Statistical analysis. Quantitative data were analysed for comparison between 2 groups using independent t-test. Results with P values of less than 5% were considered statistically significant.

RESULTS

Stem cell and Limbal markers in HTCEC and LEC

mRNA analysis of HTCEC and LEC showed they expressed stem cell markers Nanog, Oct4 and Sox2 and the limbal markers P63, C/EBPδ, BMI-1, Cytokeratin 3 (CK3), Connexin43 (Cx43) and ABCB5 (Figure 1A-D). ICC analysis of HTCEC showed that some cells expressed Nanog and ABCB5 (Figure 1E-F), no staining is seen in control (G). mRNA analysis of HTCEC for ΔNp63 isoforms showed they expressed α and β (H) but not γ (data not shown).

HTCEC SP and NSP, both expressed ABCG2 and ABCB5; with some cells showing co-expression of both (Figure 2A-C). Quantification of ABCB5 expression in both SP and NSP cells isolated from the same cell preparation (Figure 2 D-E), showed the mean signal intensity of ABCB5 expression in SP was 26.35 ± 8.70 and NSP was 24.17 ± 8.07. The mean difference between the two groups was significantly higher in SP than NSP, *P=0.02* (supplementary Table 3).

Colony forming analysis of HTCEC SP and NSP

HTCEC SP and NSP, both formed colonies (Figure 2 F-G), but the NSP colonies were smaller and contained fewer cells The difference in the cell number/colony between the SP and NSP cell fractions was significant *P*=0.01 (Figure 2H).

Chemotactic Potential of HTCEC

ICC analysis (Figure 3A-B) and measurement of mean fluorescent intensity (supplementary Table 3) for CXCR4 expression showed both HTCEC SP and NSP expressed CXCR4. However, the NSP had a lower level of CXCR4 expression compared to the SP.

To examine CXCL12-mediated cellular migration chemotaxis experiments were performed. The same numbers of HTCEC but without addition of CXCL12 in the media were used as control (background migration). Following stimulation with 300 nM CXCL12 for 5 hours, we found HTCEC were migratory in response to CXCL12. The difference in the means of migrant cells/high power fields for HTCEC in comparison to background migration (media without CXCL12) were statistically significant (P=0.009) (Figure 3C).

Human Leucocyte Antigen (HLA)-typing and HLA expression in HTCEC

We performed HLA-typing and showed HTCEC expressed both Class I-A,B,C and Class II (HLA-DR and HLA-DQ) antigens (data not shown). We further examined HLA expression in HTCEC using FACs analysis. In unstimulated condition, there was constitutive expression of HLA Class I but very low expression of Class II antigens compared to controls. Result of FACS analysis and median fluorescence index (MFI) values are provided in (Figure 4A-B). The difference between MFI of Class I-A, B, C to control was significant (P=0.003), the differences between MFI of Class II antigens compared to control was not significant (P>0.05).

After stimulation with IFN-γ, HTCEC expressed high levels of Class I and Class II antigens (Figure 4C). The highest expression was observed for HLA-Class I and HLA-DR, followed by lower expression of HLA-DP and very low expression of HLA-DQ. After treatment with TNF-α alone, HTCEC showed low expression for all HLA antigens, with the exception of Class I which showed a slight increase in expression compared to control. When treated with

a combination of TNF-α and IFN-γ, HTCEC showed HLA expression for all antibodies which was higher than that observed when cells were treated with TNFα alone, however these levels where still lower than the levels observed when HTCEC were treated with IFN-γ alone. MFI values are provided in (Supplementary Figure 1).

DISCUSSION

We compared LEC and HTCEC at the transcriptional level and found both expressed stem cell markers and common limbal markers [6-11]. CK3, a marker for corneal epithelial differentiation was robustly expressed in the LEC but low in HTCEC, indicating that HTCEC differentiated poorly in the culture conditions we employed. We previously reported ABCB1 expressed in both HTCEC and LEC [3]. ABCB1 has been reported to contribute to the SP phenotype of ovarian cancer cells [12]. LEC and HTCEC both expressed CX43 which has been previously been reported to be expressed in LEC cells [13]. Using primers previously reported to detect the three isoforms of ΔNp63 [11] we observed that HTCEC expressed the α isoform known to be important for LSC proliferation and migration, and the β isoform but lacked expression of the γ isoform, while the latter two isoforms have previously been reported to be expressed in resting LSC they become upregulated during limbal cell differentiation [11]. ICC analysis of HTCEC showed that some cells expressed Nanog and ABCB5 and we previously reported that HTCEC SP and NSP express ABCG2, ΔNp63 (antibody used detected all 3 isoforms) and Sox2 [4], suggesting HTCEC contains stem cells. LSP cells have been reported to have stem cell characteristics such as colony formation [3, 14]. HTCEC SP and NSP also formed colonies, but the SP formed bigger colonies.

We previously reported consistent HTCEC SP yields of (0.2%), while we observed LEC SP yields varied (0.1% to 0.8%) [4]. Donor variability and quality of donor tissues are factors

known to influence corneal epithelial outgrowths [15], and these might impact SP yields from tissues.

ABCB5 plays a role in LSC maintenance and corneal wound healing [7]. In our study HTCEC and LEC expressed ABCB5, while HTCEC SP and NSP cells both expressed ABCB5 with SP having a higher expression, supporting ABCB5 as an important LSC marker [7].

We showed that HLA Class I-A, B, C could be detected in unstimulated HTCEC, while Class II antigens HLA-DR, HLA-DP and HLA-DQ expressions were low/minimal compared to control. This was similar to findings described for unstimulated human corneal epithelial cultures [16, 17]. HLA Class II expression in HTCEC was inducible by pro-inflammatory cytokines. Interferon (IFN)-γ in particular, upregulated HLA Class I-A,B,C and Class II-HLA-DR, HLA-DP and HLA-DQ expression. Induction of HLA-DR expression by IFN-γ stimulation in human corneal epithelial and endothelial cultures has been demonstrated previously [16, 18]. There is limited literature on induction of HLA-DP in non-marrow derived cells or HLA-DP-negative populations. However, our results show that HTCEC mimics the immunogenicity of human corneal epithelium [18], where HLA Class II (-DR and –DP) expression was inducible by IFN-γ treatment, and a very low but concomitant HLA-DQ expression was related to cellular differentiation.

Chemokines are important for immune cell trafficking in pathological and physiological conditions. CXCR4 expression and CXCL12 ligand secretion has previously reported in the cornea [19, 20]. We showed that HTCEC constitutively express CXCR4 and are chemotactic in response to CXCL12.

Conclusion.

We provide the first data on characterisation of ABCB5 in LSP supporting the importance of this marker as a LSC marker. Further, the presence of SP cells in the HTCEC cell line that express both ABCG2 and ABCB5 lends support to the use of the SP cell assay as a useful tool for selection of stem cells. SP HTCEC also contained a significant number of CXCR4 positive cells which may be useful for studying stem cell migration. We also provide evidence that HTCEC is in many ways comparable to LEC and is therefore suitable as a robust model for the study of LSC biology.

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AUTHOR CONFLICT OF INTEREST STATEMENT

The authors indicated no conflicts of interest.

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FIGURE LEGENDS

Figure 1. HTCEC and LEC express stem cell and limbal markers. Representative images of results of PCR analysis of HTCEC and LEC for mRNA expression of common stem cell markers (note both populations express all 3 genes) (A) and limbal stem cell markers, LEC (B) and HTCEC (C), N=5. Lane 1 p63 (143 bp), lane 2 c/ebpd (111 bp), lane 3 bmi-1 (132 bp) lane 4 cytokeratin3 (125 bp), lane 5 connexin43 (249 bp), lane 6 GAPDH (100 bp). HTCEC and LEC (4 different primary derived donor LEC) also express ABCB5 mRNA (D). GapdH was used as loading control throughout. Representative images of ICC of analysis of HTCEC show some cells expressed express Nanog (E) and ABCB5 (F) (positive cells indicated by arrows). (G) IgG-only negative control shows no staining in HTCEC, N=3. *(DAPI=blue, FITC-conjugated secondary antibody= green).* Results of PCR analysis for ΔNp63 isoforms (H), HTCEC express the α isoform (1388 bp) and the β isoform (1374 bp).

Figure 2. HTCEC SP cells express ABCG2 and ABCB5. Representative images of ICC analysis of ABCB5 and ABCG2 expression, N=3. Expression of ABCG2 (A) and ABCB5 in SP cells (B). Arrow indicates positive cells. Image overlay of ABCG2 and ABCB5 stained SP cells (C) note some cells express both transporters (indicated by arrows). ABCB5 expression in SP (D) and NSP (E) cells from the same cell preparation (*[FITC-conjugated secondary anti-mouse antibody= green, Rhodamine-conjugated anti-rabbit secondary antibody = red, Dapi –nuclear stain blue, Scale bars = 20μm]*. HTCEC SP and NSP cells have colony forming ability. Phase contrast images showing colony formation of SP and NSP-sorted HTCEC on Day 5 of culture (F) SP and (G) NSP. Number of cells per colony plotted against cell count in SP and NSP cells in HTCEC, difference in the cell number/per colony between the SP and NSP cell fractions was significant P=0.010 (H), note the NSP cells form more colonies but these contain fewer cells. Scale bar =100μm.

Figure 3. HTCEC express CXCR4 and migrate in response to CXCL12. Representative images of ICC analysis for CXCR4 expression in HTCEC, N=3. (A) Images showing expression of CXCR4 in HTCEC SP and (B) NSP (positive cells indicated by white arrows), *(DAPI=blue, FITC-conjugated anti-mouse secondary antibody= green, Scale bars = 20μm).* (C) Mean number of migrant cells/high power fields from three biological replicates for CXCL12-mediated migration unsorted HTCEC in comparison to background migration (control). Treatment with CXCL12 ligand was at 300 nM for 5 hours. *[*Difference in mean values between control and CXCL12 treated group]*.

Figure 4. FACS analysis of HLA expression in HTCEC of unstimulated and stimulated cell populations (A) Histograms showing unstained population (control) and cell populations stained with HLA Class I-A,B,C and Class II antibodies. (B) Median Fluorescence Index of HLA expression for control and stained populations without cytokines stimulation (N=3). Mean MFI for Class I was significantly different to control but not for other Class II molecules (C) Representative FACS histograms out of 3 replicates showing HLA expression of Class I and Class II antigens in HTCEC following stimulation with Interferon-γ, tumour necrosis-α and combined stimulation of both (10 ng/ml, 3 days). Cells were stained with FITC-conjugated Class I, HLA-DR, HLA-DQ antibodies and HLA-DP. Negative control for HLA-DP was secondary IgG only [*Light grey – unstimulated cells, red – stimulated cells, yellow – IgG only stimulated cells]*.

Supplementary Figure 1. Median Fluorescence Index of HLA Class I-A,B,C and Class II expression in HTCEC for control (unstimulated) and stimulated populations under cytokines

treatment at 10 ng/ml for 3 days (N=3). Treatment by (A) Interferon-γ alone (B) Tumour necrosis-α alone(C) combined treatment. Results are from two biological replicates.

Supplementary Table 1. Antibodies used in flow cytometry and ICC.

Supplementary Table 2. Oligonucleotides primers and amplification conditions.

Supplementary Table 3. Statistics for image quantification analysis (signal intensities) of CXCR4 and ABCB5 expression in limbal SP and NSP in HTCEC.