Differentiation and molecular profiling of human embryonic stem cell-derived corneal epithelial cells

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Abstract. It has been suggested that the isolation of scalable populations of limbal stem cells may lead to radical changes in ocular therapy. In particular, the derivation and transplantation of corneal stem cells from these populations may result in therapies providing clinical normality of the diseased or damaged cornea. Although feasible in theory, the lack of donor material in sufficient quantity and quality currently limits such a strategy. A potential scalable source of corneal cells could be derived from pluripotent stem cells (PSCs). We developed an *in vitro* and serum-free corneal differentiation model which displays significant promise. Our stepwise differentiation model was designed with reference to development and gave rise to cells which displayed similarities to epithelial progenitor cells which can be specified to cells displaying a corneal epithelial phenotype. We believe our approach is novel, provides a robust model of human development and in the future, may facilitate the generation of corneal epithelial cells that are suitable for clinical use. Additionally, we demonstrate that following continued cell culture, stem cell-derived corneal epithelial cells undergo transdifferentiation and exhibit squamous metaplasia and therefore, also offer an *in vitro* model of disease.

Introduction

Chronic ocular surface problems result in debilitating visual impairment and corneal stem cell dysfunction. Invariably, patients with such problems require extensive surgical procedures, which include stem cell transplantation and immunosuppressive therapy. As a result, this approach is limited and a number of other concerns exist, which include the potential for infection, the limited supply of donor tissue and the absence of a well-defined specific limbal stem cell marker. In addition, the long-term preservation of the corneal rims decreases the viability of limbal stem cells, thereby reducing the quality of the available biological material for transplantation.

An alternative source of corneal cells are pluripotent stem cells (PSCs) of embryonic origin [embryonic stem cells (ESCs)] or induced PSCs (iPSCs). PSCs can expand into large numbers, whilst maintaining their differentiation potential, promising an almost unlimited supply of human somatic cell types. Moreover, iPSCs can serve as an immunologically-matched stem cell resource which has obvious clinical application.

We developed a robust *in vitro* corneal derivation procedure from human ESCs (hESCs) and examined the molecular and cellular changes in ocular biology as differentiation ensued. hESCs, grown on collagen IV in medium containing corneal limbal fibroblast-conditioned medium and fetal calf serum, have previously been shown to express limbal epithelial markers, such as CK3 and CK12, but also CK10, a skin epithelial cell marker (1). In order to address this anomaly, the central aim of this study was to differentiate true corneal epithelial progenitor cells from human ESCs under defined conditions.

In this study, we evaluated the gene expression profiles of hESCs (H9 and RCM1) following a corneal differentiation protocol which relied on conditioned medium from human limbal fibroblasts. Crucially, both hESC lines were maintained on Matrigel without a mouse embryonic fibroblast feeder layer in serum-free growth medium (mTeSR-1; StemCell Technologies, Vancouver, BC, Canada) which creates a safer environment (lower risk of contamination or disease transmission during culture) for the differentiation of hESC-derived corneal cells with features and functions similar to limbal stem cells (LSCs) which could potentially facilitate ocular surface regeneration. In order to verify the veracity of our approach, we characterised

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the molecular profile of hESC-derived corneal cells by investigating 12 different molecular markers of corneal epithelial and limbal stem cells, but also included CK10, CK19, vimentin and α-actin, which are markers characteristic of pathological ocular conditions. We wished to determine whether the differentiation system generates healthy corneal-like epithelial cells with no pathological features, such as abnormal transdifferentiation (CK10, CK19 or vimentin expression). We also examined the expression of genes which are global markers of adult stem cells, including p63α (a marker of epithelial stem cells, which supports stem cell proliferation and regulation in epithelial cells) and ATP-binding cassette, sub-family G (White), member 2 (ABCG2; a marker of basal limbal epithelial stem cells) but also leucine-rich repeat containing G protein-coupled receptor 5 (LGR5), which has been identified as a novel stem cell marker (2) and limbal stem cell marker (3). Furthermore, the expression of paired box 6 (Pax6) (4), the master control gene for eye morphogenesis, was investigated.

Materials and methods

Isolation and culture of human limbal epithelial cells. Surplus corneal buttons were obtained from the Manchester Eye Bank, Manchester Royal Eye Hospital, Manchester, UK. All corneas were received from donors with specific consent for research purposes, as previously described (3). The corneal rings were washed with CnT-20 medium and the cells were prepared and cultured as previously described (3). Corneal epithelial cells were cultured up to passage 2 (P2) and were then assessed for molecular profiles.

Culture and differentiation of hESCs into epithelial-like cells. Both hESC (H9 and RCM1) lines were maintained on Matrigel without a mouse embryonic fibroblast feeder layer. The day after the plating of the colonies, the serum-free growth medium (mTeSR-1; StemCell Technologies) was replaced by human limbal fibroblast conditioned medium and this was changed every second day according to a previously described method (1). Differentiation was identified based on changes in the morphology of the cells, the downregulation of the expression of stem cell-specific markers [Nanog homeobox (Nanog) and octamer-binding transcription factor 4 (Oct4)], and concomitant upregulation of markers for differentiated cell types (p63, ABCG2 and cytokeratins). The visual inspection of the ESC colonies with histochemical evaluation of the cells was performed to maintain the quality control of cell cultures. The cells proceeded through the differentiation protocol and were collected at 0, 4, 10, 16 and 21 days for further investigation.

Immunocytotochemistry. The expression of selected ESC-related markers was confirmed by immunocytochemistry using antibodies directed against p63α, ABCG2 and LGR5. Immunocytochemical analysis was performed as previously described (5). Briefly, the cells were fixed in cold acetone for 10 min and then incubated overnight at 4˚C with primary antibodies, including a mouse monoclonal antibody for human p63α (1:100), a rabbit polyclonal antibody for human ABCG2 (1:50) both from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and a rabbit polyclonal antibody for human LGR5 (1:50; Abcam, Cambridge, UK). Following a 5-min wash, secondary biotinylated antibodies (Dako-Agilent Technologies, Santa Clara, CA, USA) were added at a dilution of 1:500 for 1 h at room temperature. The cells were finally counterstained with haematoxylin and then visualized and captured using a Leica DM IRB microscope.

Flow cytometry. Flow cytometry was performed as previously described (6) to determine cell differentiation within the culture and to asses any apoptotic response of the hESCs to the culture conditions. The samples were analysed using a FACSCalibur flow cytometer and the results were analysed using CellQuest Pro software (both from BD Biosciences, San Jose, CA, USA).

Total RNA isolation. RNA was isolated using TRIzol reagent (following the manufacturer's instructions) and 1 μg of RNA was reverse transcribed into cDNA using random hexamers according to the manufacturer's instructions (all reagents were obtained from Promega, Madison, WI, USA). Prior to reverse transcription (RT), total RNA was quantified using a NanoDrop ND-1000 Spectrophotometer (Labtech International, Ltd., Uckfield, UK).

Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative PCR (qPCR). In order to analyse the gene expression profiles of the cells, several genes were selected for RT-PCR analysis, using total RNA derived from the H9 and RCM1 cells before and during differentiation toward a corneal phenotype. In addition, the quantitative validation of the expression of selected genes was performed by qPCR on Custom TaqMan array plates (Applied Biosystems, Foster City, CA, USA) (n=3 for H9 and RCM1 differentiated samples at each time point).

RT-PCR reactions were performed for the differentiated H9 and RCM1 cells at each selected time point using master mix [Taq buffer, 25 mM $MgCl_2$, 10 mM dNTPs, Taq polymerase (1:5), 10 μM primers, dH_2O, cDNA (20 ng/μl)] (all reagents were from Promega). RT-PCR reactions were performed using the Veriti 96 well Thermal Cycler (Applied Biosystems), at 94˚C for 5 min, followed by 35 cycles at 94˚C for 1 min, 56˚C for 1 min, 72˚C for 1 min. The PCR products were resolved and visually analysed on a 2% agarose gel in 1X TAE (triacetate EDTA) running buffer. PCR amplicon size was estimated using a 1,000 bp DNA ladder (Promega). Primer sequences are presented in Table I.

The quantitative validation of gene expression levels was carried out by qPCR (Applied Biosystems StepOne Real-Time PCR Systems) for the H9 (n=3) and RCM1 (n=3) samples at each time point (day 0, 4, 10, 16 and 21) using custom TaqMan arrays plates, applying the Universal TaqMan PCR master mix (Applied Biosystems), following the manufacturer's instructions. Amplification was performed for each cDNA (20 ng/μl) sample in triplicate and the gene expression level was normalized to GAPDH. The fold change in expression of the target gene relative to the internal control gene (GAPDH) at various time points was assessed. qPCR data were presented as the fold change in gene expression normalised to an endogenous reference gene and relative to the undifferentiated control (day 0). In our time course assessment of gene expression, the calibrator sample represents the amount of transcript that is

Table I. Human primer sequences used for reverse transcription-polymerase chain reaction (RT-PCR).

Gene name	Forward primer	Reverse primer	Product size (bp)
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	50
Pax6	AACAGACACAGCCCTCACAAACA	CGGGAACTTGAACTGGAACTGAC	275
P63α	ATGCAGTACCTTCCTCAGCACACA	TTCCTGAAGCAGGCTGAAAGGAGA	104
ABCG2	GTGCACATGCTTGGTGGTCTTGTT	AGCTCGGTCTTAACCAAAGGCTCA	159
LGR5	CTCTTCCTCAAACCGTCTGC	GATCGGAGGCTAAGCAACTG	181
CK3	AAGAACATGGAGGACCTGGTGGAA	AGGCACTGTCCACATCCTTCTTCA	115
CK12	AGGCCATGGATCTCTCCAACAACA	TTCCAACACTGGAAGCAGACATGC	123
CK10	TTGGTGGAGGTAGCTTTCGTGGAA	AGAAGGCCACCATCTCCTCCAAAT	130
CK18	GCATCCAGAACGAGAAGGAG	ACTTTGCCATCCACTATCCG	1027
CK19	TTGTCCTGCAGATCGACAAC	TCTTCCAAGGCAGCTTTCAT	514

expressed at time zero/day zero of differentiation. Primers for TaqMan arrays were designed by Applied Biosystems.

Statistical analysis. For statistical analysis, we used the SPSS software package. Statistical significance of the results was assessed by the analysis of variance (ANOVA) with Bonferroni correction to evaluate the differences between means, as presented in Table II. Results were considered significant at $p<0.05$ and are presented in Table II.

Results

Using this model, we initially determined the molecular profiles of cultured limbal cells and those of ESC-derived corneal-like cells.

Molecular profile of cultured limbal epithelial cells. Human limbal epithelial cultures were successfully established in feeder-free and serum-free CnT-20 culture medium (Fig. 1a and b). Conventional RT-PCR profiles of cultured corneal limbal epithelial cells (n=4) demonstrated the mRNA expression of the putative stem cell markers, ABCG2 and p63α. The expression of p63α in all 4 cultures was detected at a low level. However, strong complex patterns of expression of CK3 and CK12 indicated the existence of the very specific corneal epithelial cell phenotype. The expression of both sets of markers suggests that the limbal cell cultures are heterogeneous, containing both stem cells and differentiated epithelial cells. In addition, there was a strong expression of Pax6, the molecular marker which is a key requirement for eye morphogenesis, which regulates cell proliferation and determines differentiation (Fig. 1c). On the basis of these results, we decided to use a feeder-free and serum-free environment for hESC differentiation in the subsequent experiments.

Molecular profile of hESCs which differentiated into corneal-like cells. The differentiation of hESCs was performed on Matrigel without a mouse embryonic fibroblast feeder layer with epithelial conditioned medium (Fig. 2). The differentiation of hESCs into corneal epithelial-like cells was analysed by qPCR or conventional RT-PCR, flow cytometry and immunocytochemistry. RNA obtained from the differentiated hESCs for PCR analysis was of very high quality (260/280, 260/230 in the range of 1.89-2.0). The molecular profile of selected cytokeratins with GAPDH as an internal control was examined in corneal cells which differentiated from RCM1 cells (Fig. 3). CK3 and CK12 expression was very weak, although CK3 expression increased from the second day of differentiation; the expression of CK10, CK18 and CK19 was abundant from the early stages of differentiation. In addition, the quantitative validation of the expression levels of selected genes was carried out in both the H9 and RCM1 cells using

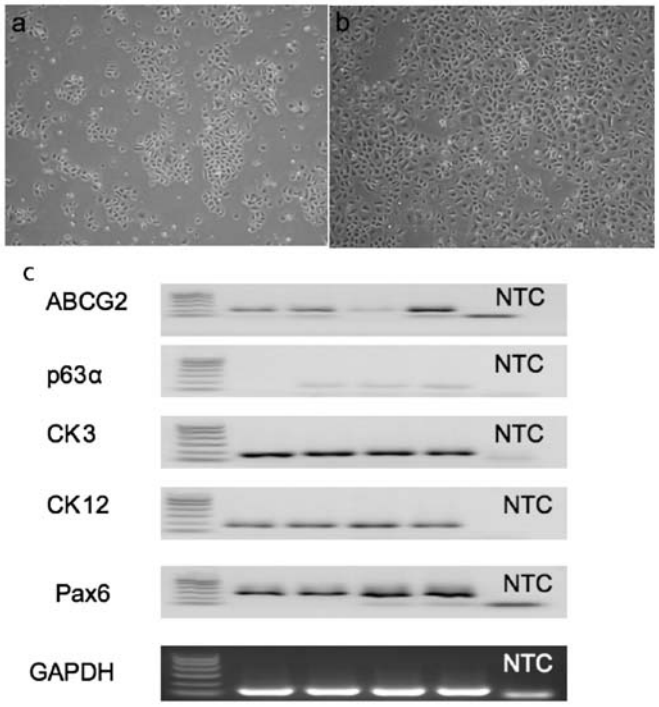

Figure 1. Representative images from 4 experiments of cultured corneal epithelial cells at (a) day 6 and (b) day 8 in culture. Cells were seeded at 200,000/25 cm^2 and cultured up to passage 2 (P2) in feeder-free and serum-free CnT-20 culture medium and were then assessed for molecular profile. (c) Conventional RT-PCR profiles of cultured corneal limbal epithelial cells (representative experiment of n=4) demonstrated mRNA expression of the putative stem cells markers, ABCG2 and p63α, and markers for an advanced state of corneal epithelial differentiation, cytokeratins CK3 and CK12, as well as Pax6, the molecular marker which regulates cell proliferation and determines differentiation.

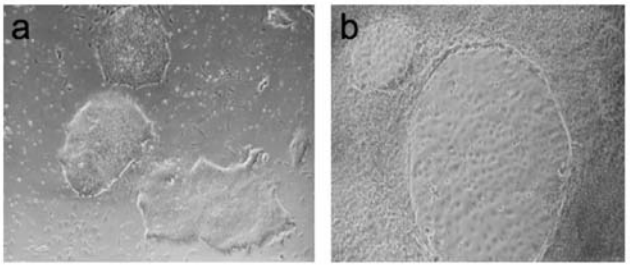

Figure 2. Representative images from differentiation of RCM1 cells into corneal-like epithelial cells at (a) day 1 and (b) day 21 in culture. Differentiation of hESC was performed on Matrigel without a mouse embryonic fibroblast feeder layer with epithelial conditioned medium. Differentiation medium was added at day 1, 24 h after splitting.

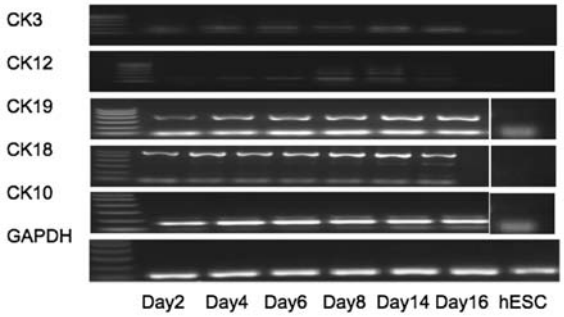

Figure 3. Conventional RT-PCR molecular profile of differentiated RCM1 cells. Expression of selected cytokeratins (CK3, CK12, CK19, CK18 and CK10) with GAPDH as an internal control was examined in differentiated RCM1 cells over time. Representative images from four experiments.

custom TaqMan array plates, which confirmed the findings of conventional RT-PCR for the selected genes of interest. The expression levels of p63α, Pax6, ABCG2, CK4, CK18, CK19, vimentin and α-actin mRNA were quantified with GAPDH as an internal control. In addition, the expression of the hESC markers, Nanog and Oct4 was examined at all time points during the differentiation of hESCs (Table II).

qPCR analysis revealed that the expression of the hESC markers, Nanog and Oct4, declined significantly over the 21 days of the differentiation protocol in both the RCM1 and H9 cell lines. However, these markers remained detectable at the end of the differentiation protocol, which suggests that there was a proportion of cells which had not undergone differentiation after 21 days (Table II). The quantitative analysis of the expression of Pax6 in the RCM1 and H9 cell lines showed that the differentiation process was characterised by a moderate (although not significant) expression of Pax6 at day 4 and by a significantly increased expression by day 10 compared with day 0 (undifferentiated cells). Pax6 expression decreased from day 10 onwards; however, the expression was still maintained at a statistically significant level (Table II).

Pax6 expression was accompanied by a significantly high expression of the nuclear transcription factor, p63α (a marker of epithelial stem cells, which supports stem cell proliferation and regulation in epithelia) at day 10 (RCM1 cell line) and day 16 (H9 cell line). The abundant expression of p63α was detected in both cell lines until the final day of the differentiation protocol (Table II). In addition, there was a very abundant expression of ABCG2 (a marker of basal limbal epithelial stem cells), which showed a similar pattern to p63α expression in both cell lines with a significant increase at day 10 (RCM1 cell line) and day 16 (H9 cell line) (Table II). Thus, in view of these results, the differentiation model we developed provides hESC-derived cells with specific signatures of early epithelial progenitor cells.

Furthermore, the quantitative analysis of the mRNA encoding global markers of adult stem cells was accompanied by a significantly increased expression of CK19 and vimentin in both cell lines, which confirms the analysis from conventional RT-PCR, where CK10 was found to be abundantly expressed. Vimentin expression was statistically significant at day 10 of differentiation, while CK19 expression reached significance at day 16. Additionally, we investigated α-actin expression, which was also significant at day 10 in both cell lines. These results may indicate that differentiated cells, upon prolonged culture conditions, undergo transdifferentiation and represent a pathological phenotype of squamous metaplasia. Furthermore, we detected the expression of CK4 and CK18, both components of the stratified squamous epithelium. However, only CK18 showed a statistically significant expression at day 16 (RCM1 cell line) and day 10 (H9 cell line) (Table II).

All results from qPCR are presented as a fold change relative to day 0 and were calculated using the ΔCt method (e.g., a decrease in Ct by 1 reflects a 2-fold increase in expression, a decrease by 2 reflects a 4-fold increase).

Immunocytochemical staining of differentiating hESCs revealed the expression of the putative stem cell markers, ABCG2, p63 and LGR5, which was sustained during the subsequent stages of the differentiation process (data not shown) and confirmed the findings from qPCR, indicating the existence of side-population cells with a stem-cell phenotype. The expression of LGR5, at day 0 and during differentiation was confirmed by flow cytometry and indicated that LGR5 can be considered as an additional stem cell marker (Fig. 4). The results from flow cytometric analysis of the expression of Pax6, LGR5, CK3 and CK12 in differentiating hESCs are presented in Table III. In the undifferentiated RCM1 and H9 cell lines, Pax6 expression was very low. By day 4 of differentiation, the expression of Pax6, although still low, had increased by 3.4- and 5.9-fold in the RCM1 and H9 cell lines, respectively; however, it presented a decrease in expression at day 6 and 10 in the RCM1 and H9 cell lines, respectively. On day 16, the H9 cells once again showed an upregulated Pax6 expression, although this returned to the control levels by day 21; in the RCM1 cells, from day 6 to 21, the expression of Pax6 decreased, but not to the control levels. There was a concomitant increase at day 4 in the frequency of positive cells, as shown for the H9 cells (Fig. 4), from 20 to 55% which was maintained until day 16 but which fell below the control levels by day 21.

LGR5 expression in the undifferentiated RCM1 cells was much higher than that in the H9 cells, and was upregulated at day 4 of differentiation; however, its expression was lower than that of the control cells at days 6 and 21. In the H9 cells, the expression of LGR5 was reduced on days 4 and 10, but recovered to the control levels by day 16 and was upregulated at day 21. The frequency of positive cells mirrored these

Table II. qPCR validation of selected targets.

Gene symbol	Cell line	Time-point: Day 0 FC ± SD	Day 0 p-value	Day 4 FC ± SD	Day 0/4 p-value	Day 10 FC ± SD	Day 0/10 p-value	Day 4/10 p-value	Day 16 FC ± SD	Day 0/16 p-value	Day 4/16 p-value	Day 10/16 p-value	Day 21 FC ± SD	Day 0/21 p-value	Day 4/21 p-value	Day 10/21 p-value	Day 16/21 p-value
NANOG	RCM1	1.0±0.002	-	0.5±1.5	-	0.3±0.15	<0.05	-	0.1±0.37	<0.05	-	-	0.4±0.5	<0.05	-	-	-
	H9	1.0±0.1	-	0.2±1.6	-	0.1±0.2	<0.005	-	0.1±0.3	<0.05	-	-	0.1±0.4	<0.05	-	-	-
OCT	RCM1	1.0±0.05	-	0.5±0.002	-	0.2±0.45	-	-	0.2±0.001	<0.005	-	-	0.2±0.5	-	-	-	-
	H9	1.0±0.02	-	0.3±0.1	<0.05	0.1±0.1	-	<0.05	0.0±0.07	<0.05	-	-	0.0±0.1	<0.05	<0.005	<0.001	-
Pax6	RCM1	1.0±0.7	-	10.5±1.7	-	27.5±0.4	<0.005	-	11.6±0.005	<0.005	-	-	12.9±0.45	<0.05	-	-	-
	H9	1.0±0.2	-	2.6±1.9	-	42.7±0.05	<0.05	-	63.7±0.07	-	-	-	32.9±0.4	<0.05	-	-	-
p63α	RCM1	1.0±0.01	-	18.8±3.15	-	22.9±0.3	<0.05	-	731±0.005	<0.001	<0.001	<0.05	303±0.37	<0.01	-	<0.05	<0.05
	H9	1.0±0.2	-	1.94±0.01	-	3.9±0.2	-	-	68.8±0.2	<0.001	<0.005	<0.05	127.6±0.3	<0.001	-	<0.05	<0.05
ABCG2	RCM1	1.0±0.3	-	17.8±0.9	-	7.9±0.2	<0.05	-	42.2±0.0	<0.05	-	<0.05	23.0±0.2	<0.05	-	-	-
	H9	1.0±0.2	-	2.1±0.9	-	1.1±0.2	-	-	13.6±0.3	<0.05	-	<0.05	11.3±0.2	<0.005	-	-	<0.005
Vimentin	RCM1	1.0±0.1	-	4.8±0.7	-	8.3±0.2	<0.05	-	2.2±0.0	<0.05	-	<0.05	4.7±0.3	-	-	-	-
	H9	1.0±0.1	-	4.5±0.8	-	4.9±0.1	<0.001	-	5.5±0.0	<0.005	-	-	12.0±0.2	<0.005	-	-	<0.05
α-actin	RCM1	1.0±0.2	-	4.6±0.6	-	5.9±0.2	<0.001	-	2.2±0.0	-	-	-	4.9±0.1	<0.05	-	-	<0.05
	H9	1.0±0.0	-	10.7±1.3	-	13.7±0.2	<0.05	-	3.3±0.3	<0.005	-	-	3.7±0.1	<0.005	-	<0.05	-
CK18	RCM1	1.0±0.0	-	0.5±0.1	-	1.0±0.0	-	-	6.8±0.0	<0.001	<0.005	<0.005	5.7±0.0	<0.005	<0.005	<0.005	-
	H9	1.0±0.05	-	0.3±0.9	-	0.3±0.2	<0.05	-	1.3±0.0	<0.05	-	-	1.4±0.0	<0.005	-	-	-
CK19	RCM1	1.0±0.4	-	4.7±0.1	-	3.0±0.0	-	-	11.7±0.0	<0.05	<0.05	<0.005	24.2±0.0	<0.05	<0.01	<0.001	<0.001
	H9	1.0±0.1	-	1.0±0.1	-	1.6±0.1	-	-	12.5±0.1	<0.005	<0.005	<0.005	4.6±0.2	-	-	-	<0.05

Data are shown as fold change (Bonferroni corrected p-value for differences between means). FC, fold change; SD, standard deviation.

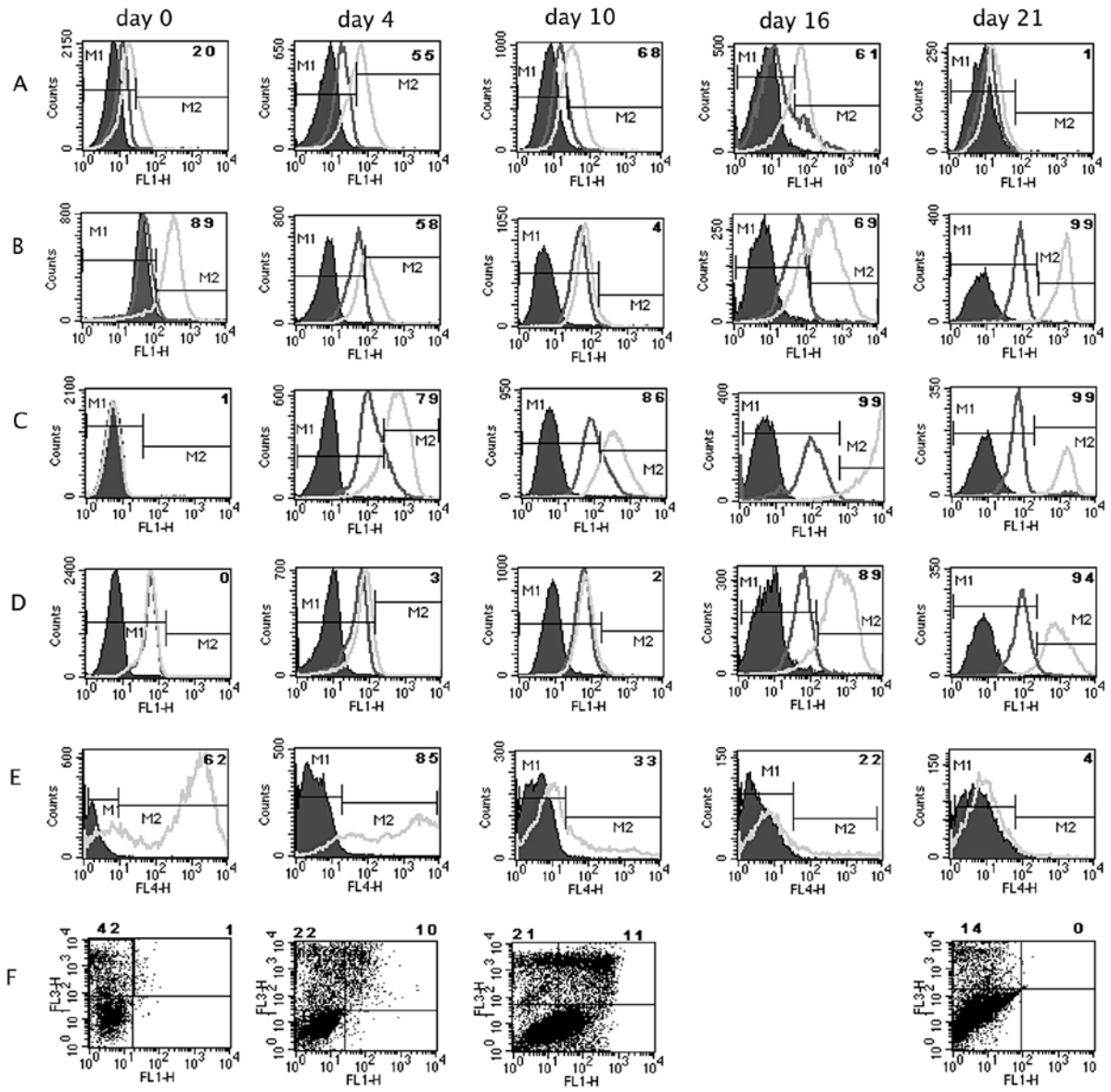

Figure 4. Flow cytometric analysis of the expression of (A) Pax6, (B) LGR5, (C) CK3, (D) CK12 and (E) TRA-1-60 in the differentiating H9 cell line at days 0, 4, 10, 16 and 21. Results are presented as the percentage of live cells: unstained control (filled histogram), 2˚ antibody control (dark grey) and stained cells (light grey). (F) Dot plots of propidium iodide (FL3) vs. Annexin V (FL1) indicate the percentage of live, dead and apoptotic cells at days 0, 4, 10 and 21.

changes, falling from day 0 to 10m then increasing on day 21 (Table III).

Although no CK3 expression was detected in the H9 control cells, the subsequent pattern of expression of CK3 was compared in both the RCM1 and H9 cell lines, increasing by day 4 compared with the control cells. In the RCM1 cells, the expression of CK3, which had increased by 5.2-fold on day 4, fell by day 6 and then returned to the control levels on day 21. By contrast, in the H9 cells, CK3 expression was downregulated on day 10, peaked on day 16, and its expression was still 3-fold higher on day 21 than it had been on day 4. The frequency of CK3-positive cells increased from 1% on day 0 to 79% by day 4 and rose to 99% by day 21 (Fig. 4).

The expression of CK12 in the RCM1 cells was similar to that of CK3, increasing by day 4 (2.2-fold), then falling from day 6 to below that of the control cells by day 21. In the H9 cells, the pattern of CK12 expression, although lower, was similar to that of CK3 (Table III). No expression was detected in the control cells. At day 4, a relatively low level of CK12 expression was observed (0.5-fold) which fell by day 10 then was increased on day 16 (29.8-fold) and day 21 (39-fold) as compared to day 4 (Table III). The frequency of positive cells was <3% up to day 10, and then rose to 89% (day 16) and 94% (day 21) (Fig. 4), concomitant with its expression patterns.

Staining with TRA-1-60 was included to monitor the differentiation status of the hESC lines. At day 0 and 6, >80% of the RCM1 cells were TRA-1-60-positive; however, by days 16 and 21 this fell to approximately 20%, indicating that undifferentiated hESCs remained in the cultures (Table III). The frequency of TRA-1-60-positive H9 cells declined throughout the differentiation period from 85% in the controls to 4% on day 21.

Table III. Expression of molecular markers as assessed by flow cytomertry.

A, RCM1 cell line

	Day of differentiation				
	0	4	6	16	21
MFI					
Pax6	14	48	0	na	20
LGR5	1470	4017	480	na	342
CK3	1823	9609	5073	na	1636
CK12	980	2235	1326	na	363
TRA-1-60	81.97	na	89.46	21.01	16.88
% Positive cells					
Annexin V^+/PI^-	12.86	na	na	29.97	47.57
Annexin V^+/PI^+	19.96	na	na	0.15	1.25

B, H9 cell line

	Day of differentiation				
	0	4	10	16	21
MFI					
Pax6	5	29	9	21	2
LGR5	259	14	14	281	1408
CK3	0	418	263	9569	1266
CK12	0	14	7	417	546
TRA-1-60	2127	304[a]	103[a]	170[a]	174[a]

Data are presented as mean fluorescent intensity (MFI) calculated as peak channel value of stained cells minus the peak channel value of unstained cells for Pax6, LGR5, CK3 and CK12 or as the percentage of positive cells for TRA-1-60 and Annexin V/PI staining. [a]Median values used for MFI calculation. na, not assessed.

Staining of the RCM1 cells for signs of apoptosis indicated that before the initiation of differentiation, <15% of the cells were in early apoptosis (Annexin V^+/PI^-) and 20% were in late apoptosis (Annexin V^+/PI^+). In the later stages of differentiation, although the frequency of cells in late apoptosis had decreased to <5%, the proportion of cells in early apoptosis had increased to 30% at day 16, and by day 21 almost 50% of the cells were Annexin V^+/PI^- (Table III). By contrast, in the H9 cells, no early apoptotic cells were observed at any time point. The frequency of Annexin V^+/PI^- cells fell from 42% at day 0 to 14% at day 21 and only approximately 10% of late apoptotic cells were observed on day 4 and 10 (Fig. 4).

TRA-1-60 and Annexin V/PI staining suggested that the differentiation and survival potential of the H9 cells was superior to that of the RCM1 cells, which by day 21 were mostly undifferentiated or apoptotic. In addition, the decreased expression of corneal epithelial cell markers at day 21 suggested that the corneal-like epithelial phenotype of the differentiated cells was altered from this time point onwards.

Discussion

The aim of this study was to develop a protocol for the differentiation of corneal epithelial cells from hESCs and characterise the molecular changes during this differentiation. Our results demonstrated that the applied differentiation model on Matrigel, without the use of a mouse embryonic fibroblast feeder layer, led to the differentiation of hESCs into corneal-like epithelial cells, which upon prolonged cell culture, underwent transdifferentiation and exhibited the pathological features of squamous metaplasia. To our knowledge, this study describes, for the first time, the complex molecular characterisation of hESC differentiation toward a corneal epithelial-like phenotype through the analysis of 12 different molecular markers which belong to three groups: stem cell-related markers (LGR5, ABCG2 and p63), cytokeratins, which are markers for an advanced state of corneal epithelial differentiation (CK3, CK12, CK4 and CK18) and markers of pathological changes (CK10, CK19, α-actin and vimentin). This study provides a robust model with which to investigate corneal differentiation and to develop strategies that allow the rescue of cells from abnormal cell differentiation/metaplasia.

During the course of differentiation, we detected the expression of Pax6, which plays a pivotal role during eye morphogenesis and ocular surface development, but is also important for post-natal development and maintenance of the adult cornea (4). The Pax6 protein is localised only in epithelial cells of the cornea (7) and has been described as a gene promoter for CK12 expression (8). Thus, it is the most important molecular marker for the characterisation of differentiated corneal cells. The expression of Pax6 was detected by qPCR and flow cytometry (3- and 5-fold increase in RCM1 and H9 cell lines, respectively) during the early stages of the differentiation process at day 4. By day 10, we observed a statistically significant increase in Pax6 expression in both cell lines by qPCR, which was sustained at a significant level at the subsequent time points compared with day 0. However, flow cytometric analysis revealed a detectable but reduced signal at the endpoint of the differentiation process. The observed co-expression of CK12/Pax6 in the differentiated cells is consistent with what is known to occur during ocular development (9). Therefore, the data presented in this study strongly suggest that our *in vitro* differentiation protocol mimics the processes of ocular cell development *in vivo* (10) and the existence of the proper pathway of corneal cellular differentiation. Moreover, if there was a lack of Pax6 expression during differentiation then we could conclude that the cells were following a default differentiation pathway and differentiating into epidermis (11). It has been reported that deficiencies in Pax6 expression lead to impaired corneal epithelial function and progressive ocular surface failure (e.g., aniridia), which may be due to an altered niche development (12). The downregulation of Pax6 has been linked to the abnormal epidermal differentiation of corneal epithelial cells during squamous metaplasia (13). Therefore, the robust expression of Pax6 during hESC differentiation has to be considered a key factor in controlling the phenotype of ocular epithelial-like cells.

In this study, Pax6 expression was accompanied by the expression of the nuclear transcription factor, p63α, which has been suggested to be a putative marker of limbal epithelial stem

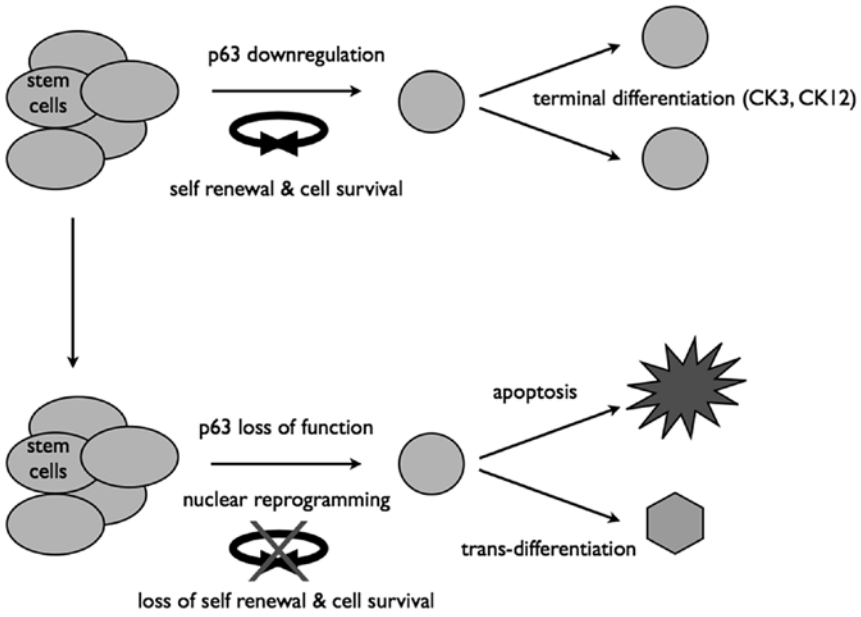

Figure 5. Schematic showing that in corneal progenitor cells, p63 promotes proliferation, self-renewal and maintains 'stemness'. In the absence of p63, stem cells are not able to proliferate and self-renew and either die by apoptosis or undergo abnormal transdifferentiation through nuclear reprogramming. Alternatively, pre-existing terminally differentiated cells may also transdifferentiate.

cells (14). The expression of p63 has been suggested as an identification marker of early epithelial progenitor cells sustaining a proliferative potential (15,16). In our study, we detected a significantly high expression of p63 by qPCR at day 10 (RCM1 cell line) and day 16 (H9 cell line), which was confirmed by immunostaining. Thus, the applied differentiating protocol led to the generation of proliferating epithelial progenitor cells with features of self-renewal. *In vivo* the p63 transcription factor is located in the limbal basal epithelium and it has been suggested that stem cells expressing p63α actively proliferate and migrate to the central cornea to restore the corneal epithelium. However, during the course of this migration, the cells progressively lose p63α expression and switch to the expression of p63β and p63γ, which regulate terminal differentiation, and co-express CK3/CK12 (markers of terminal differentiation). In cultured limbal epithelial cells, the expression of p63α was weak, indicating that the cultured cells were possibly gaining the profile of transit amplifying cells, which can be detected in the central cornea. These cells appeared to be proceeding toward a fully differentiated corneal epithelial cell phenotype which was confirmed by the strong co-expression of CK3 and CK12 (Fig. 1c). In this study, we observed CK3 and CK12 expression during the early stages of corneal differentiation in both the ESC lines. We also observed Pax6/p63/CK3- and CK12-positive cells, which suggests the existence of the correct pathway of limbal epithelial cell derivation from hESCs in the early stages of differentiation and confirms the correlation between Pax6 and CK12 expression. Furthermore, we analysed the ABCG2 expression pattern in both cultured corneal epithelial cells and in hESCs differentiated to a corneal fate. ABCG2 has been suggested to be a universal marker of stem cells. In putative limbal epithelial cells, this protein is localised to the membrane and cytoplasm and it is thought that ABCG2-positive cells have higher colony-forming efficiency values *in vitro* (17). We detected a significantly high ABCG2 expression by qPCR at day 10 (RCM1 cell line) and day 16 (H9 cell line) during the course of differentiation. This was confirmed by immunostaining at day 10 of differentiation. This expression was coincident with p63 expression. We also detected the expression of LGR5 (orphan G protein-coupled receptor), which has been detected in many types of organ-specific stem cells and has been suggested as a general molecular marker of stem cell populations (18). We have previously identified LGR5 expression in corneal limbal cells (3) and during the differentiation of hESCs to a corneal fate; however, in this study, LGR5 expression was absent from the fully differentiated H9 and RCM1 cells. Flow cytometric analysis revealed that both cell lines expressed LGR5 prior to differentiation (RCM1>H9). In the H9 cells, LGR5 expression was reduced initially and then returned to the initial levels at day 16, before increasing by 5.44-fold at day 21. The frequency of positive cells followed the same pattern, falling from 89% at day 0 to 4% by day 10 and rising to 99% by day 21. Data from previous studies, as well as ours support the hypothesis that LGR5-positive cells are associated with cellular stemness and that they may be important during embryogenesis. For example, it has been reported that LGR5-deficient mice undergo early neonatal death due to many malformations (19). This molecular marker appears to control important aspects of stem cell behavior. LGR5 was identified as a Wnt target gene (20) and this signalling pathway appears to act synergistically with Notch signalling in limbal cell self-renewal and daughter cell commitment to differentiation.

In this study, the detection of the stem-cell-related markers, p63 and ABCG2, at day 10 and LGR5 from the beginning of the differentiation process indicates that this differentiation process provides cells with specific signatures of early epithelial progenitor cells. Upon further culture, these cells undergo terminal differentiation into corneal-like epithelial cells characterised by CK3 and CK12 expression. However, following continued culture, analysis of the differentiating cells indicates

the presence of features consistent with a pathological phenotype, squamous metaplasia (13). Biochemically, squamous metaplasia is characterised by the loss of the corneal-specific keratins, CK3 and CK12, and by the upregulation of CK1 and CK10. In this study, the decreased and weak expression of CK3 and CK12 (Table III) was accompanied by the expression of CK10 (Fig. 3) and by the significantly high expression of CK19 (at day 16) and vimentin (at day 10) in both cell lines (Table II). In addition, α-actin expression was also significantly high at day 10 in both cell lines (Table II). This co-expression pattern of CK10, α-actin and vimentin may mark the abnormal cell differentiation associated with transdifferentiation and metaplasia. The normal corneal epithelium consists of a non-keratinising squamous epithelium. However, the inflamed corneal and conjunctival tissues can deviate from their normal expression and may show abnormal cytokeratin profiles. Thus, the corneal epithelium that displays metaplastic changes expresses CK19 (a marker of conjunctival epithelium) and CK10 (a feature of dermal epithelium). In eye pathology, chronic ocular surface inflammatory conditions are known to cause keratinisation or metaplasia of the conjunctival and corneal epithelium. These chronic conditions can also lead to corneal limbal stem cell deficiency. It has been suggested that loss of limbal stem cells through various injuries and pathological processes may result in migration of conjunctival-derived cells to the corneal surface. Therefore, the presence of conjunctival-derived cells (CK19-positive cells) and goblet cells has been interpreted as a feature of limbal stem cell deficiency. In addition, vimentin expressino has been detected in a variety of limbal dysplastic lesions (21) but, alternatively, vimentin expression has also been reported as a feature of corneal regeneration and has been found in the epithelium of keratoconus specimens (22).

Comparing the expression of corneal-epithelial markers by flow cytometry, it appeared that in undifferentiated cells, Pax6 and LGR5 expressino was low (H9<RCM1), whereas CK3 and CK12 were expressed in the RCM1 cells but absent from H9 cells. Early in the differentiation process, the expression of these makers was modulated in both cell lines. In the RCM1 cells, the expression peaked by day 4, then fell to or below the initial levels. The expression of Pax6, CK3 and CK12 peaked in the H9 cells by day 16 and LGR5 peaked on day 21. CK3 and CK12 expression remained above starting levels on day 21, while Pax6 expression returned to baseline levels. The frequency of H9 cells positive for LGR5, CK3 and CK12 cells reflected the modulation of expression and remained maximal on day 21, when the frequency of PAX6-positive cells returned to 0%. These differences between cell lines may be a result of the starting expression of corneal epithelial markers. Initial levels were high in the RCM1 cells; therefore, they may have peaked early, then adapted over time in culture; indeed their TRA-1-60 staining was weak at the initiation of culture. In the H9 cells, initial weak staining may have resulted in them peaking later, having downregulated TRA-1-60 on >95% of cells and showing little if any apoptosis.

The observation of the abnormal cell phenotype associated with metaplasia following on from successful differentiation to a corneal fate is supported by the Annexin V/PI data (Table III). This clearly shows that in the later stages of culture following differentiation, the cell population comprises mainly apoptotic and undifferentiated hESCs rather than corneal-like cells. This accounts for the decreased expression of corneal epithelial cells markers and for the expression of markers which are typical of pathological conditions, such as squamous metaplasia. This process may be explained by the development of oxidative stress during prolonged cell culture conditions in serum-free and protein-free media. In addition, it has been postulated that in the absence of p63, stem cells are unable to proliferate and self-renew, and therefore die by apoptosis resulting in poor or absent tissue regeneration (23).

In conclusion, we induced the differentiation of cells which co-expressed Pax6 and limbal stem cell markers (p63, ABCG2 and LGR5), indicating the specific signature of early epithelial progenitors proceeding to a corneal phenotype (CK3 and CK12). However, in the later stages of culture, there was a significant decrease in p63 and an increase in CK10 expression, indicating the existence of oxidative stress. This loss of p63 leads to abnormal transdifferentiation and apoptosis provoked by a change in the cellular environment (24). This concept has been described in eye pathology (25) and confirmed in this study by altered expression of CK10, α-actin and vimentin in epithelial corneal cells (3) and now in differentiated cornea-like epithelial cells derived from hESCs (Fig. 5). Our results clearly indicate that the applied differentiation protocol leads to the development of cells with a corneal epithelial phenotype which, with continued culture, undergo transdifferentiation and metaplasia and present features of abnormal cells. The system provides the possibility of differentiating pluripotent cells into corneal cells both for disease modelling and, in the longer term, particularly with the advent of induced pluripotent cell technologies, as a source of cells for transplantation and clinical intervention.

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