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Identification and *In Vitro* Expansion of Buccal Epithelial Cells

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Abstract

Ex vivo-expanded buccal mucosal epithelial (BME) cell transplantation has been used to reconstruct the ocular surface. Methods for enrichment and maintenance of BME progenitor cells in *ex vivo* cultures may improve the outcome of BME cell transplantation. However, the parameter of cell seeding density in this context has largely been neglected. This study investigates how varying cell seeding density influences BME cell proliferation and differentiation on tissue culture polystyrene (TCPS). The highest cell proliferation activity was seen when cells were seeded at 5×10^4 cells/cm^2. Both below and above this density, the cell proliferation rate decreased sharply. Differential immunofluorescence analysis of surface markers associated with the BME progenitor cell population (p63, CK19, and ABCG2), the differentiated cell marker CK10 and connexin 50 (Cx50) revealed that the initial cell seeding density also significantly affected the progenitor cell marker expression profile. Hence, this study demonstrates that seeding density has a profound effect on the proliferation and differentiation of BME stem cells *in vitro*, and this is relevant to downstream cell therapy applications.

Keywords

cell differentiation, cell proliferation, cell transplant, cheek and mouth mucosa, epithelial cell

Introduction

Worldwide, corneal disease is the most common cause of blindness[1]. Severe ocular surface damage may be caused by thermal and chemical burns and microbial infection, may result from autoimmune disease (e.g. Stevens–Johnson syndrome), or arise congenitally (aniridia-associated keratopathy)[2,3]. Ocular surface disease develops as a consequence of limbal stem cell dysfunction. The limbus, the junctional area between the transparent cornea and the opaque sclera with its overlying conjunctiva, is believed to contain the self-renewing adult stem cells responsible for normal corneal epithelial regeneration. It has been shown that transplantation of limbal stem cells can be used as a therapeutic intervention in those with severe ocular surface disease[4,5]. Donor tissue can be taken from the normal contralateral eye, but this can compromise the integrity of the normal eye[5]. In many instances, limbal stem cell deficiency is bilateral, requiring the use of allogeneic donor tissue and immunosuppression. Furthermore, the number of limbal stem cells present in the graft may be insufficient to retain graft viability. Therefore, other sources of stem cells are highly desirable. Grafts of buccal mucosa are sometimes used as an alternative to cells taken from the eye[6–9]. Extensive studies have been conducted to investigate the possibility of using buccal mucosal epithelial (BME) cells as donor tissue, since harvesting those cells avoids invasive surgery required for cells from the limbus[2,9]. Previous studies have confirmed that BME progenitor cells share common characteristics with limbal stem cells and that there is overlap in progenitor marker expression between these populations[10].

Cultures of BME cells have been used to prepare grafts that can restore corneal defects due to limbal stem cell

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malfunction[2,7], but outcomes related to the technique used to prepare the graft still remain to be evaluated[11]. An essential prerequisite of epithelial grafts for long-term restoration of the ocular surface is the presence of cells possessing specific progenitor cell characteristics within the graft tissue. However, there is no definitive marker for BME progenitors, so isolation of an enriched progenitor population using standard cell sorting cytometric techniques is unfeasible. Additionally, during *in vitro* culture, BME stem cells inevitably undergo differentiation and lose their stem cell phenotype. A parameter that has largely been overlooked in studying BME cell behaviour *in vitro* is the cell seeding density. The published literature would suggest that intercellular contacts and paracrine signalling are essential for retaining cellular homeostasis in the stem cell microenvironment[12,13]. We speculate that BME progenitor cells may rely on those cues for optimal expansion and maintenance of their properties. These microenvironmental effects in turn depend on the initial cell seeding density. An understanding of this behaviour is highly relevant to clinical applications in BME progenitor cell expansion and delivery to treat ocular surface disease[14].

In this study, we investigated BME progenitor cell behaviour *in vitro* in respect to initial cell seeding densities, to reveal the changes in BME progenitor cell proliferation and markers that take place during culture. To evaluate the influence of cell seeding density on cell behaviour, isolated epithelial cells were seeded at 2×10^4, 5×10^4 and 15×10^4 cells/cm^2. The differentiation trajectory of the cell population was tracked during culture by monitoring changes in expression of p63[15–19], cytokeratin19 (CK19)[16–18], ATP-binding cassette subfamily G, member 2 (ABCG2)[20,21] and cytokeratin 10 (CK10)[22–28]. Connexin 50 (Cx50), expressed in the transient amplifying cell population of the cornea but absent from the stem cell population of the limbus[29–31], was included in the panel as a potential means to distinguish progenitor populations within the BME cell cultures.

Materials and Methods

BME cell isolation

Rat buccal mucosa tissue was obtained from the oral cavities of 100 g female Wistar rats (n=4, from the Animal Care Unit, South Australia Health and Medical Research Institute, Adelaide, Australia). We isolated buccal tissue from animals under complete anaesthesia based on the guidelines approved by the South Australia (SA) Pathology Animal Ethics Committee. Oral epithelial cells were isolated from the tissue according to previous established protocols with some modification[32]. In brief, tissue was washed several times with sterile phosphate-buffered saline (PBS) containing 100 U/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL amphotericin B. Samples were then incubated in 2 mg/mL dispase (Sigma Aldrich, NSW, Australia) in MCDB 153 medium (Sigma Aldrich) at 37°C and 5% CO_2 for 1 h. The epithelial layer was removed from submucosal connective tissue. The separated epithelial strips were incubated for 10 min in warm (37°C) 0.05% w/v trypsin and 0.02 mM ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich). The epithelial layer was then gently disaggregated with a needle and micropipette tip in trypsin-EDTA for 5 min to yield a single cell suspension. After addition of MCDB 153 medium containing 10% foetal bovine serum (FBS) (Sigma Aldrich), the cell suspension was centrifuged for 5 min at 200 *g*. The supernatant was discarded and cells were resuspended in complete MCDB 153 medium: MCDB 153 medium containing 50 pM triiodothyronine (T3) (Sigma Aldrich), 10 ng/mL beta nerve growth factor (R&D Systems), 200 ng/mL hydrocortisone, 5 ng/mL epithelial growth factor (Jomar Bioscience, SA, Australia), 5% insulin-transferrin-selenium (ITS) liquid medium supplement (Sigma Aldrich) and 5% FBS and were incubated in a humidified atmosphere of 5% CO_2 at 37°C. Thereafter, 3×10^4 viable freshly isolated cells were deposited on glass slides by centrifugation at 400 *g* for 3 min using a Cytospin 4 system (ThermoFisher Scientific, Waltham, MA, USA).

Proliferation studies

BME cells isolated as described above were seeded at densities of 2×10^4, 5×10^4 and 15×10^4 cells/cm^2 into CellCarrier 96 well plate (PerkinElmer, Waltham, MA, USA). The seeded BME cells were then cultured for a period of 5 h, 24 h, 48 h, 72 h. 96 h, 120 h and 144 h in complete MCDB 153 medium at 37°C in a humidified atmosphere containing with 5% CO_2. To evaluate cell adhesion and proliferation, the media was removed and cells were fixed with 3.7% paraformaldehyde in PBS at room temperature for 15 min then washed two times with PBS before permeabilisation with 0.25% Triton X100 in PBS for 5 min at room temperature. Following three washes in PBS, cells were subsequently stained with 100 µL Hoechst 33342 (2.0 µg/mL, Invitrogen, Carlsbad, CA, USA) in PBS for 15 min at room temperature. Following washing with PBS, cells were imaged with an Operetta high-content imaging system (PerkinElmer). Images were analysed and cell numbers were quantified through nuclei using Harmony software (PerkinElmer).

Cell doubling time was calculated by online software Doubling Time Computing (available from: http://www.doubling-time.com/compute.php). Cell proliferation was also evaluated with a tetrazolium-based colorimetric assay (XTT assay kit; Sigma-Aldrich, Saint Louis, MO, USA), according to the manufacturer's instructions. Briefly, cells were seeded at seeding densities of 2×10^4, 5×10^4 or 15×10^4 cells/cm^2 into flat-bottomed 96-well plates in a final volume of 100 µL of culture medium per well. After the incubation period, 50 µL of the XTT labelling mixture was added to each well. The cells were further incubated for 4 h at 37°C in a humidified atmosphere containing 5% CO_2 to allow XTT formazan production. The absorbance was measured with a microplate reader at a wavelength of 450 nm.

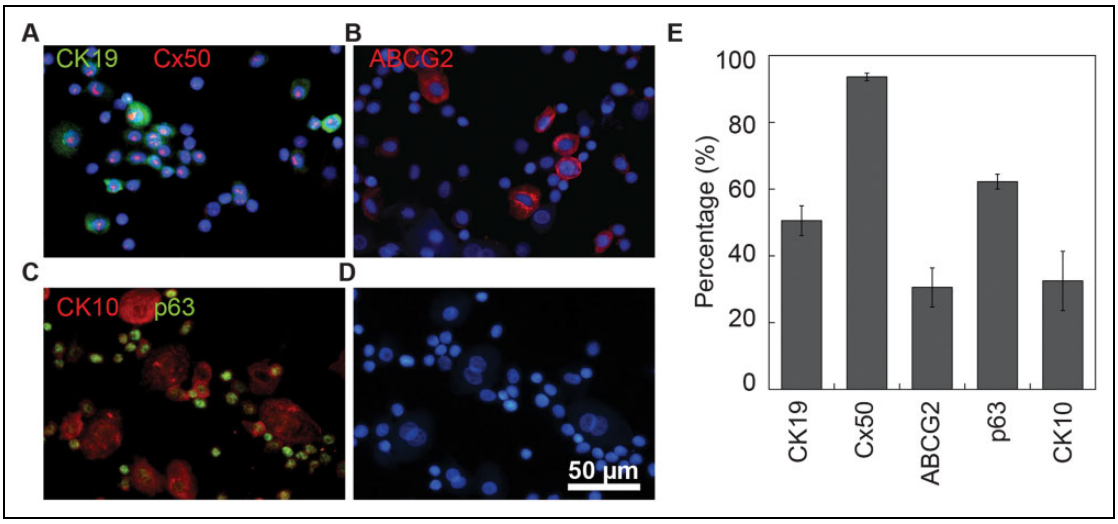

Fig. 1. Immunofluorescence staining of freshly isolated BME cell suspensions, revealing the expression of proteins associated with differentiation and progenitor status before seeding on TCPS. (A) Expression of CK19 (labelled green) and Cx50 (labelled red) were visualised using immunocytochemistry. (B) ABCG2 surface expression (red). Blue signals indicate nuclei of epithelial cells which were stained using Hoechst 33342. (C) Isolated BME cells were labelled for p63 (green) and CK10 (red) expression. (D) p63/CK10 labelled population shown in (C) counterstained with Hoechst 33342. (E) Quantification of marker protein expression in epithelial cells after isolation and prior to seeding on TCPS. The error bars represent the standard error. n=4.
BME: buccal mucosal epithelial; TCPS: tissue culture polystyrene.

Immunofluorescence of epithelial cells

The cytospin smears and epithelial cells cultured on TCPS were fixed with 4% paraformaldehyde for 10 min and were permeabilised with 0.25% Triton X100 for 5 min at room temperature. After washing with sterile PBS without Ca^{2+} and Mg^{2+}, cells were treated with blocking solution of 10% serum from the species in which the secondary antibody was raised for 30 min to block non-specific binding of the primary antibodies. Samples were then incubated with the selected primary antibody at 4°C overnight. Primary antibodies were specific for p63 (1:200, rabbit polyclonal immunoglobulin (Ig)G, H-137), CK19 (1:100, goat polyclonal IgG, N-13), ABCG2 (1:200, goat polyclonal IgG, D-20), CK10 (1:200, goat polyclonal IgG, K-14), and Cx50 (1:200, mouse monoclonal IgG, B-11). All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Positive and negative controls of immunofluorescence experiments were included to support the validity of staining and identify experimental artefacts. After washing the cells three times in PBS for 5 min each wash, cells were incubated with secondary antibody at room temperature for 60 min. Secondary antibodies were donkey anti-rabbit (FITC), donkey anti-goat (FITC), donkey anti-goat (PE), donkey anti-mouse (FITC) and donkey anti-mouse (CFL 555) at a dilution of 1:200 in PBS containing 1% bovine serum albumin. Counterstaining was performed with Hoechst 33342. Marker expression levels (% positive cells) were assessed by identifying cell nuclei and scoring the co-localised marker expression (nuclear or cytoplasmic as appropriate) as positive or negative. Immunofluorescence analysis was repeated in four independent experiments for each marker. Marker expression scores of a subset of differentiation and stem cell markers were verified using the Operetta high-content imaging system (PerkinElmer). Nuclei were detected using the 4',6-diamidino-2-phenylindole (DAPI) channel and each nucleus was identified as an object representing a cell. The cytoplasmic region was defined around each nucleus using either fluorescence in the PE or FITC channels. Cell fate and extent of differentiation were obtained by dividing the number of identified positive cells by the total number of cells. Analysis was performed after isolation and incubation time of 5, 24, 48, 72, 96, 112, and 144 h on TCPS.

Statistical analysis

The significance of differences observed between the numbers and proportions of cell populations attached on microarray spots were determined by analysis of variance followed by Tukey's posthoc test. $P<0.05$ was regarded as significant.

Results

Analysis of stem cell marker expression in BME populations after isolation

In order to establish a baseline for comparison with later *ex vivo* cultures, the progenitor content of fresh BME isolate was assessed by immunocytochemistry. The expression of a panel of several cell markers consisting of proteins associated with progenitor epithelial populations (CK19, ABCG2, p63) and differentiated epithelial cells (CK10) was used to assess the identity of the freshly isolated epithelial cell suspensions before seeding on TCPS (Fig. 1). Freshly isolated

cells were deposited on glass slides by centrifugation for immunofluorescence analysis.

CK19, a progenitor marker in both the limbus and buccal mucosal epithelium[26,33,34] was observed in 51 ± 5% of isolated cells. A high proportion of the isolated cell population (62 ± 2%) expressed p63. Expression of p63 is also associated with progenitor populations in both the buccal mucosa and limbus and was used as a predictor of long-term corneal regeneration after transplant of cultivated epithelial cell sheets[15–19]. ABCG2 is expressed in a subset of $p63^+$ cells in the limbus, and is proposed to define the stem cell population from transient amplifying progenitor cells[20,21]. It has been reported to mark cell populations in cultivated BME cell sheets. ABCG2 was detected in 31 ± 6% of the cell population. CK10, known to be present in differentiated suprabasal cells within multilayered epithelium[22–28], was employed as a negative selection marker. CK10 was expressed by 33 ± 9% of isolated cells. Together, this analysis showed that a progenitor/stem-cell-containing population was successfully isolated from oral mucosal tissue.

Interestingly, the majority of isolated cells (93 ± 1%) of epithelial cells expressed Cx50. Cx50, a gap junction constituent, is expressed within all layers of the corneal epithelium but is excluded from the limbus[29,30], where the most primitive stem cells are thought to reside. Hence it is a potential marker to differentiate stem cells from transient amplifying cell progeny[31]. In this context, and distinct from the expected peripheral localisation, Cx50 expression was noted to be entirely localised to the nucleus.

Seeding density influences cell proliferation and stem cell marker retention in BME cell cultures

In order to understand the influence of seeding density on *ex vivo* BME cultures, the isolated epithelial cells from the oral cavities of four rats were seeded in quadruplicate at initial densities of 2×10^4, 5×10^4 and 15×10^4 cells/cm^2 onto TCPS. Initially cells were allowed to attach to TCPS surfaces for 5 h before observation. While in the literature, incubation times from 10 min to overnight have been cited for incubation of BME on the substrate[2,35,36], we observed that a 5 h incubation time provided enough time for cells to adhere on the surface; and that this incubation time was suitable to obtain a high fraction of cells expressing stem cell markers[37]. Cell attachment varied directly with the initial cell seeding density (Fig. 1). At the lowest cell density tested, 2×10^4 cells/cm^2, very few cells (around 710 cells/cm^2) attached to TCPS, which was significantly ($P\leq0.005$) lower than for the other initial cell seeding densities. The images presented in Fig. 2A show that cell proliferation was most successful at an initial density of 5×10^4 cells/cm^2. By comparison, seeding at lower cell density (2×10^4 cells/cm^2) or higher cell density (15×10^4 cells/cm^2) gave reduced proliferation after 96 h in culture. As seen in Fig. 2B, the proliferation rate of the BME cells on the TCPS substrate varied considerably, depending on the seeding density. At cell seeding densities of 5×10^4 and 15×10^4 cells/cm^2, cell numbers increased 2.5-fold and 1.5-fold, respectively, after 144 h in culture, while for cells at the lowest cell seeding density, there was no appreciable change in cell number during 144 h in culture. The maximum proliferation rate was attained at an initial seeding density of 5×10^4 cells/cm^2 with a doubling time of 45 h. At seeding densities higher and lower than 5×10^4 cells/cm^2, the proliferation rate decreased. Under the same culture conditions, the doubling time for initial cell seeding density of 2×10^4 cells/cm^2 was 140 h and for the highest cell seeding density of 15×10^4 cells/cm^2, the doubling time was 74 h. The cell number reached a maximum at 72 h for cultures seeded at a density of 15×10^4 cells/cm^2 and at 96 h for cultures seeded at 5×10^4 cells/cm^2. Proliferation of the cultured cells was also evaluated by XTT assay (Fig. 2C). The findings correlated with cell counting.

We next investigated whether, in addition to influencing the rate of cell proliferation, cell seeding density would affect the differentiation of BME cells during culture *via* paracrine signalling and other forms of cell–cell communication. An analysis of surface marker expression indicative of stemness or differentiation was carried out each day with the same panel of markers used to assess the BME isolate (Figs. 3–5). Expression of the stem cell and progenitor markers CK19, ABCG2 and p63 was down-regulated during the observation period for all seeding densities tested (Figs. 3 and 4). However, comparison of the expression of these markers over the 144 h time period revealed changes in the rate of cell differentiation of BME cells that were dependent on the initial seeding density of these cultures. For example, an inverse correlation between initial seeding density and expression of CK19 was noted. A rapid decline in CK19 expression was already evident 5 h after seeding at the highest cell density tested (15×10^4 cells/cm^2). CK19 expression remained around 10% higher in cultures seeded at 2×10^4 cells/cm^2 compared with cultures seeded at 15×10^4 cells/cm^2 throughout the culture period (Fig. 4A). Likewise, ABCG2 expression was retained at a higher level in cultures seeded at 2×10^4 cells/cm^2 than those seeded at higher densities ($P\leq0.01$ from 24 h to 144 h), resulting in 50 ± 3.5% ABCG2 positive cells at the end of culture, 30% higher than the proportion of ABCG2 positive cells in the other cultures (Fig. 4B). For cultures seeded at 15×10^4 cell/cm^2, CK19 and ABCG2 expression was confined to rare small cell clusters sporadically distributed across the cultured population. Immunocytochemical analysis showed these markers to be expressed more homogeneously across the cultures seeded at 2×10^4 cell/cm^2 cells (Fig. 5D, E). The expression profile of the differentiated epithelial cell marker CK10 also indicates a correlation between cell density and differentiation: throughout the culture period, the proportion of CK10 positive cells in cultures seeded at higher densities exceeds those seeded at 2×10^4 cells/cm^2 by greater than 10%.

In contrast with ABCG2 and CK19, expression of p63 was best maintained using an intermediate cell density

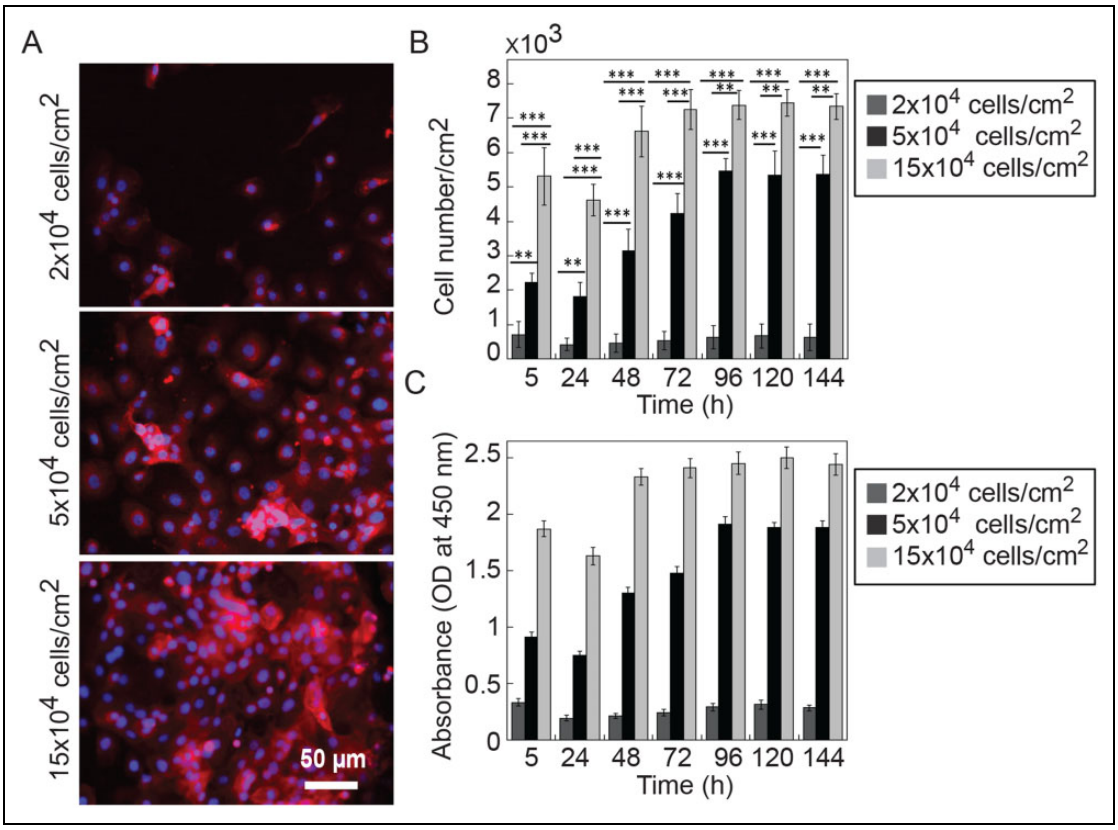

Fig. 2. The effects of initial cell density on proliferation of BME cells. (A) Fluorescence staining of BME cell after 96 h *in vitro*, cytoplasm was stained with Phalloidin (red) and nuclear was stained with Hoechst 33342 (blue). The initial seeding density is shown left of image. (B) BME cell number on TCPS from 5 h to 144 h incubation time at 37°C, 95% humidity and 5% CO_2. Cells were seeded at different density of 2×10^4, 5×10^4 and 15×10^4 cells/cm^2. (C) Cell proliferation was measured by XTT assay. Error bars show the standard error of cell counts. $n=4$, a = $P\leq0.01$, b = $P\leq0.005$.
BME: buccal mucosal epithelial; TCPS: tissue culture polystyrene.

(5×10^4 cells/cm^2). Little difference in p63 expression was seen[15,16] between cultures seeded at 2×10^4 cells/cm^2 and 15×10^4 cells/cm^2. In addition to stem cells[38], p63 is also expressed by young early transient amplifying cells in the buccal epithelium[10]. The increase in p63 expression noted in cultures seeded at 5×10^4 cells/cm^2 compared with cultures seeded at higher and lower densities might reflect the greater proportion of partially differentiated progenitor cells in these populations.

Cx50 expression was down-regulated during the culture period at all seeding densities. Expression in cultures seeded at 15×10^4 cells/ cm^2 rapidly decreased from $87\pm3\%$ to $33.5\pm4\%$ after 144 h. However, cells seeded at 2×10^4 and 5×10^4 cells/cm^2, retained Cx50 expression at $71\pm4\%$ and $70\pm6.5\%$ of the BME cell population, respectively, after 144 h in culture. Similar to the pattern observed in freshly isolated BME cells, Cx50 was again observed to localise almost entirely to the cell nucleus across all time points and culture conditions. Loss of Cx50 expression was noted in cells spatially isolated from other cells (Fig. 5A). The down-regulation of Cx50 expression more closely reflected trends seen in ABCG2 and CK19 than CK10, suggesting Cx50 levels are indicative of progenitor content, rather than differentiation, in these BME cultures.

Discussion

The transplant of *ex vivo* cultivated buccal epithelial cells is now an established means to treat limbal stem cell deficiency, with treated patient cohorts numbering in the hundreds[14,39,40]. Increased interest in this technique has led to the recent establishment of a rat animal model to optimise surgical parameters[41]. In this study, we describe how adherence to TCPS may be used to isolate and propagate progenitor cell-rich populations from rat BME cells. We demonstrate that TCPS, the conventional substrate for laboratory tissue culture, is able to support robust cell proliferation, without necessitating the use of feeder layers. This work therefore contributes to the current drive in the field to obtain defined conditions for tissue engineered epithelial cell sheets[9,42,43].

BME cells were observed to attach to TCPS surfaces within 5 h. Based on surface marker expression of CK19, ABCG2 and p63, the proportion of stem cells in this

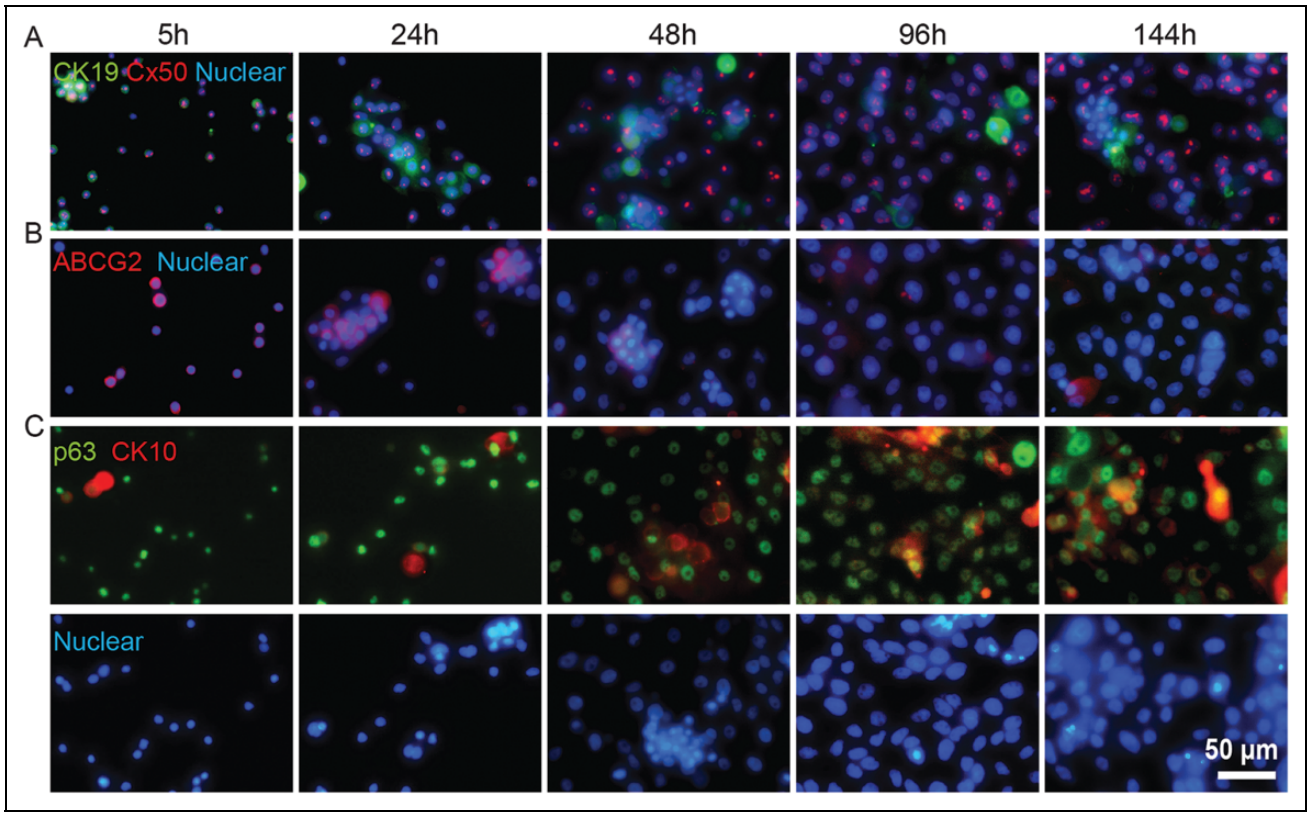

Fig. 3. Immunofluorescence staining of cultured BME cells against stem cell and differentiation markers at intervals during 144 h culture. BME cell cultures were seeded at a density of 5×10^4 cells/cm^2. Expression of the marker panel was probed at 24 h intervals after the first day of culture. (A) CK19 (green) and Cx50 (red), (B) ABCG2 (red), (C) p63 (green) and CK10 (red); counterstained panel is shown in second row. Cell populations were counterstained using Hoechst 33342.
BME: buccal mucosal epithelial.

population adherent on the surface of the TCPS at this time was higher compared with freshly isolated cells, indicating a positive selection effect. BME cells on TCPS after 5 h incubation also contained fewer CK10 positive cells compared with freshly isolated cells. This suggests that the progenitor cell population can be partially enriched through selective adhesion to TCPS. Similar results were observed in previous studies in which limbal stem cells were partially purified from mixed cell populations based on attachment to a collagen-based substrate[31].

Continued culture of the BME isolate on TCPS revealed that the extent of growth and the doubling time of BME cells varied depending on the initial cell density. A dependence of cell proliferation on the cell seeding density has been shown in prior studies with other primary cells[37,44–47]. The increased rate of proliferation observed when seeded at higher densities may be associated with transition to differentiation, which is supported by the higher proportion of differentiated cells appearing under these conditions. A minimum seeding density (greater than 2×10^4 cells/cm^2) was required to initiate cell proliferation. Little or no net growth is often observed below a critical initial cell density[12,13,48], which has been attributed to a paucity of intracellular contacts and paracrine signals[12]. Previous work comparing the cultivation of canine oral mucosal epithelial sheets at different densities on microporous cell culture inserts noted that cell sheet integrity and stratification was optimal when cultures were seeded at a similar density ($4–5\times10^4$ cells/cm^2)[43].

Subsequently, immunofluorescence staining was employed to examine how the marker expression profile of the BME cell populations varied as culture on TCPS progressed, since changes in differentiation rate in response to initial cell seeding density have also been described for other cell types such as human umbilical vein endothelial cells and mesenchymal stem cells[12,49,50]. We examined both putative progenitor (CK19, ABCG2 and p63) and differentiation markers (CK10 and Cx50). Upon comparing the BME cell marker expression profiles with respect to cell seeding density during 144 h culture, significant differences in the expression of markers were observed. The expression of the BME progenitor cell marker ABCG2 when cells were seeded at very low density was 4.3-fold and 2.5-fold higher than when cell seeding density was 5×10^4 and 15×10^4 cells/cm^2, respectively. The deviation in differentiation trajectory between cultures seeded at 2×10^4 cells/cm^2 and those

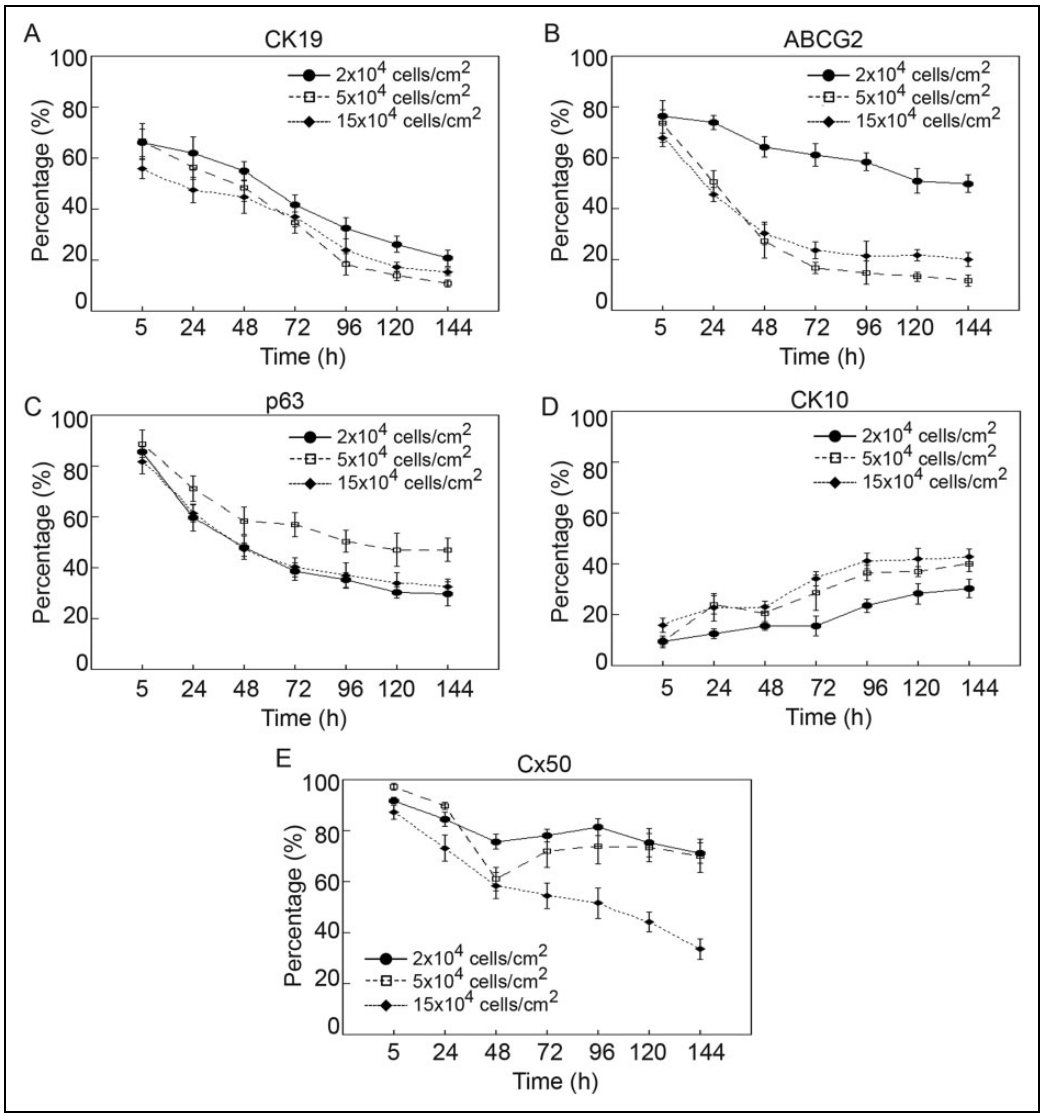

Fig. 4. Effect of initial cell seeding density on epithelial cell marker expression over the course of 144 h. (A) The expression level of CK19. (B) The expression level of ABCG2. (C) The expression level of p63. (D) The expression level of CK10. (E) The expression level of Cx50. Cells were plated at densities of 2×10^4, 5×10^4 and 15×10^4 cells/cm^2. Cells were stained for the specific markers and subsequently quantified by high-content microscopy. The error bars represent the standard error. $n=4$.

seeded at higher densities was evident from early in the culture. In several cancer cell models, ABCG2 positive cells have been noted to be tolerant of low seeding density[35,51]; conversely, ABCG2 expression is increased at low cell densities[52]. The initial cell seeding density affected CK19 expression in a similar manner to ABCG2, but the influence of density was less marked. Expression of the differentiated cell marker CK10 also indicated that stem cells are best retained at low seeding densities. In all three cell density groups, the percentage of CK10 positive cells increased with time in culture, indicating differentiation is present even at the lowest seeding densities, where growth appears almost arrested.

The retention of progenitor markers at low cell densities may reflect the limited capacity of epithelial stem cells to participate in intercellular signalling[53]. In light of this, both the high proportion of cells expressing Cx50 and the nuclear localisation of this protein were intriguing. While Cx50 has been used as a negative selection marker for limbal epithelial stem cells[33], there are no direct data comparing the expression of Cx50 in BME cell cultures. In our hands, Cx50 negative cells occurred as a very rare population in low density cultures, and only in cells that did not contact other BME cells in these cultures. Cx50 is typically localised to the plasma membrane where gap junction channels are formed between cells, but has also been reported in the nucleus[54]. Connexins have been revealed to possess a number of functions in addition to gap function intercellular communication, which include growth regulation and differentiation control[55]. Nuclear expression of Cx50 has recently

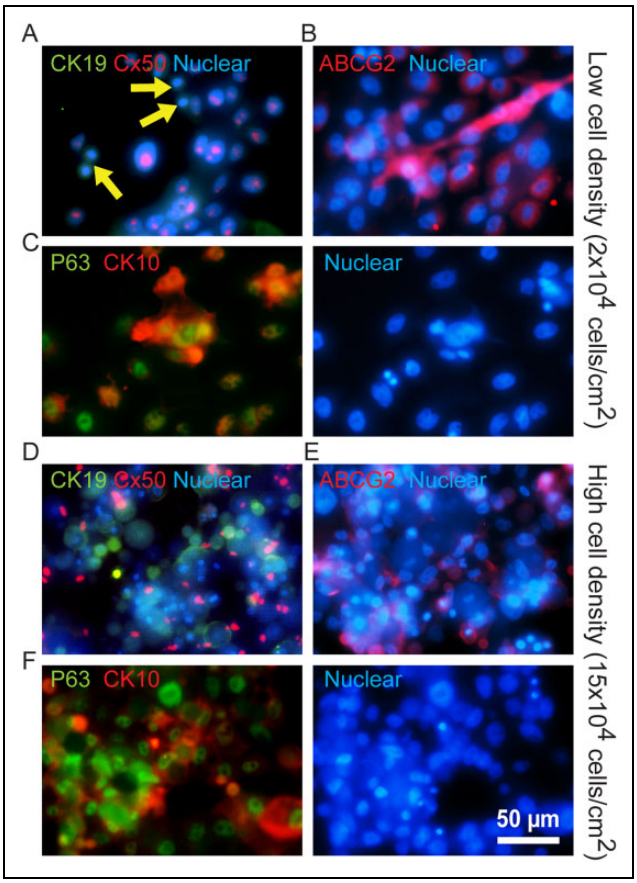

Fig. 5. Epithelial cell marker expression is dependent on initial seeding density. In all panels, blue signal marks the nuclei of epithelial cells, stained with Hoechst 33342. (A) Cells were plated at 2×10^4 cells/cm^2 and cultured for 96 h, then were stained against CK19 (green) and Cx50 (red). Yellow arrows mark loss of Cx50 expression in cells that are spatially isolated and possess limited cell contact. (B) Cells were plated at 2×10^4 cells/cm^2 and cultured for 96 h, then were stained against ABCG2 (red). (C) Cells were plated at 2×10^4 cells/cm^2 and cultured for 96 h, then were stained against p63 (green) and CK10 (red). Corresponding Hoechst 33342 staining is shown in the right panel. (D) Cells were plated at 15×10^4 cells/cm^2 and cultured for 96 h, then were stained against CK19 (green) and Cx50 (red). (E) Cells were plated at 15×10^4 cells/cm^2 and cultured for 96 h, then were stained against ABCG2 (red). (F) Cells were plated at 15×10^4 cells/cm^2 and cultured for 96 h, then were stained against p63 (green) and CK10 (red). The corresponding nuclear staining is shown in the right panel.
Cx50: connexin 50.

been reported in ependymal stem cells, which lose expression upon differentiation[56]. We observed a similar down-regulation of Cx50 expression in cultures with higher levels of differentiation (as assayed by CK10 content).

In summary, surface marker analysis across the course of the 6-d culture indicated that lower cell seeding densities were beneficial for the retention of stem cell characteristics in the BME cell population. However, cell proliferation in cultures seeded at the lowest density trialled (2×10^4 cells/cm^2) was limited. Transplantable *ex vivo* cultured BME grafts must form a confluent monolayer of cells, ideally featuring a high progenitor component to facilitate a successful graft. In this culture system, high cell proliferation must be balanced against maintenance of the progenitor cell population. A seeding density of 5×10^4 cells/cm^2 both retains high stem cell marker expression, particularly of p63, and promotes robust growth.

To conclude: a range of 'microenvironment'-related features must be optimised to ensure maintenance of BME stem cells properties for cell therapy. This study confirms that cell density is a controlling factor of BME cell proliferation and differentiation. Controlling the initial cell seeding density may enhance cell proliferation, thus potentially leading to differentiation. Here, we have demonstrated that seeding densities of 5×10^4 and 15×10^4 cell/cm^2 appeared to be associated with enhanced BME cell proliferation. Cells seeded within this seeding density range also demonstrated pronounced cell differentiation as confirmed by immunofluorescence staining of the cell markers. However, at a low cell seeding density of 2×10^4 cell/cm^2 poor cell proliferation but strong maintenance of stem cell markers was observed. Therefore, initial cell seeding density should be considered to investigate the effect of microenvironment on cell behaviour. The results of this study provide useful information with regard to the optimisation of tissue engineering protocols for culturing epithelial cells.

Ethical Approval

Approval was obtained from the institutional animal care and use committee at SA pathology (114A/12).

Statement of Human and Animal Rights

This study was carried out according to the recommendations of guidelines for laboratory animal care and safety from the Australia Health and Medical Research Institute and University of South Australia.

Statement of Informed Consent

The Statement of Informed Consent is not applicable in this study.

Declaration of Conflicting Interests

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References

1. Dua HS, Joseph A, Shanmuganathan V, Jones R. Stem cell differentiation and the effects of deficiency. Eye. 2003;17(8):877–885.
2. Nakamura T, Inatomi T, Sotozono C, Amemiya T, Kanamura N, Kinoshita S. Transplantation of cultivated autologous oral mucosal epithelial cells in patients with severe ocular surface disorders. Br J Ophthalmol. 2004;88(10):1280–1284.

3. Ebrahimi M, Taghi-Abadi E, Baharvand H. Limbal stem cells in review. J Ophthalmic Vis Res. 2009;4(1):40–58.
4. Sangwan VS, Basu S, Vemuganti GK, Sejpal K, Subramaniam SV, Bandyopadhyay S, Krishnaiah S, Gaddipati S, Tiwari S, Balasubramanian D. Clinical outcomes of xeno-free autologous cultivated limbal epithelial transplantation: a 10-year study. Br J Ophthalmol. 2011;95(11):1525–1529.
5. Dickinson A, Lako M, Kolli S, Figueiredo F. Outcomes of ex vivo expanded limbal stem cell transplantation in humans. Cytotherapy. 2013;15: S44–S45.
6. Inatomi T, Nakamura T, Koizumi N, Sotozono C, Yokoi N, Kinoshita S. Midterm results on ocular surface reconstruction using cultivated autologous oral mucosal epithelial transplantation. Am J Ophthalmol. 2006;141(2):267–275.
7. Sotozono C, Inatomi T, Nakamura T, Koizumi N, Yokoi N, Ueta M, Matsuyama K, Miyakoda K, Kaneda H, Fukushima M. Visual improvement after cultivated oral mucosal epithelial transplantation. Ophthalmology. 2013;120(1):193–200.
8. Nakajima R, Kobayashi T, Kikuchi T, Kitano Y, Watanabe H, Mizutani M, Nozaki T, Senda N, Saitoh K, Takagi R. Fabrication of transplantable corneal epithelial and oral mucosal epithelial cell sheets using a novel temperature-responsive closed culture device. J Tissue Eng Regen Med. 2015;9(5): 637–640.
9. Kolli S, Ahmad S, Mudhar HS, Meeny A, Lako M, Figueiredo FC. Successful application of ex vivo expanded human autologous oral mucosal epithelium for the treatment of total bilateral limbal stem cell deficiency. Stem Cells. 2014;32: 2135–2146.
10. Krishnan S, Iyer GK, Subramanian K. Culture & characterisation of limbal epithelial cells & oral mucosal cells. Indian J Med Res. 2010;131:422–428.
11. Menzel-Severing J, Kruse FE, Schlötzer-Schrehardt U. Stem cell–based therapy for corneal epithelial reconstruction: Present and future. Can J Ophthalmol. 2013;48(1):13–21.
12. Heng BC, Bezerra PP, Preiser PR, Alex Law S, Xia Y, Boey F, Venkatraman SS. Effect of cell-seeding density on the proliferation and gene expression profile of human umbilical vein endothelial cells within ex vivo culture. Cytotherapy. 2011; 13(5):606–617.
13. Bitar M, Brown RA, Salih V, Kidane AG, Knowles JC, Nazhat SN. Effect of cell density on osteoblastic differentiation and matrix degradation of biomimetic dense collagen scaffolds. Biomacromolecules. 2007;9(1):129–135.
14. Utheim TP. Concise review: transplantation of cultured oral mucosal epithelial cells for treating limbal stem cell deficiency—current status and future perspectives. Stem Cells. 2015;33(6):1685–1695.
15. Yang A, Kaghad M, Wang Y, Gillett E, Fleming MD, Dötsch V, Andrews NC, Caput D, McKeon F. p63, a p53 homolog at 3q27–29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Mol Cell. 1998;2(3):305–316.
16. Prakash D, Rai A, Patne S, Kumar S, Maurya O, Chandra A. Identification of human limbal stem cell by p63, a specific stem cell marker in cadaveric limbal tissue and in in-vitro limbal stem culture. J Community Health Manag. 2015;2:33–42.
17. Rama P, Matuska S, Paganoni G, Spinelli A, De Luca M, Pellegrini G. Limbal stem-cell therapy and long-term corneal regeneration. N Engl J Med. 2010;363:147–155.
18. Priya C, Arpitha P, Vaishali S, Prajna N, Usha K, Sheetal K, Muthukkaruppan V. Adult human buccal epithelial stem cells: identification, ex-vivo expansion, and transplantation for corneal surface reconstruction. Eye. 2011;25(12):1641–1649.
19. Blanpain C, Fuchs E. p63: revving up epithelial stem-cell potential. Nat Cell Biol. 2007;9(7):731–733.
20. Priya CG, Prasad T, Prajna NV, Muthukkaruppan V. Identification of human corneal epithelial stem cells on the basis of high abcg2 expression combined with a largen/c ratio. Microsc Res Tech. 2013;76(3):242–248.
21. de Paiva CS, Chen Z, Corrales RM, Pflugfelder SC, Li DQ. ABCG2 transporter identifies a population of clonogenic human limbal epithelial cells. Stem cells. 2005;23(1):63–73.
22. Merjava S, Neuwirth A, Tanzerova M, Jirsova K. The spectrum of cytokeratins expressed in the adult human cornea, limbus and perilimbal conjunctiva. Histol Histopathol. 2011;26(3): 323–331.
23. Böer-Auer A, Jones M, Lyasnichaya OV. Cytokeratin 10-negative nested pattern enables sure distinction of clonal seborrheic keratosis from pagetoid Bowen's disease. J Cuta Pathol. 2012;39(2):225–233.
24. Simanainen U, McNamara K, Gao YR, McPherson S, Desai R, Jimenez M, Handelsman DJ. Anterior prostate epithelial AR inactivation modifies estrogen receptor expression and increases estrogen sensitivity. Am J Physiol Endocrinol Metab. 2011;301: E727–E735.
25. Koike T, Yasuo M, Shimane T, Kobayashi H, Nikaido T, Kurita H. Cultured epithelial grafting using human amniotic membrane: the potential for using human amniotic epithelial cells as a cultured oral epithelium sheet. Arch Oral Biol. 2011;56(10): 1170–1176.
26. Izumi K, Terashi H, Marcelo C, Feinberg S. Development and characterization of a tissue-engineered human oral mucosa equivalent produced in a serum-free culture system. J Dent Res. 2000;79(3):798–805.
27. Xiong X, Zhao Y, Zhang W, Xie W, He S. In vitro engineering of a palatal mucosa equivalent with acellular porcine dermal matrix. J Biomed Mater Res A. 2008;86(2):544–551.
28. Poumay Y, Pittelkow MR. Cell density and culture factors regulate keratinocyte commitment to differentiation and expression of suprabasal K1/K10 keratins. J Investig Dermatol. 1995;104:271–276.
29. Matic M, Petrov IN, Chen S, Wang C, Wolosin JM, Dimitrijevich SD. Stem cells of the corneal epithelium lack connexins and metabolite transfer capacity. Differentiation. 1997;61: 251–260.
30. Dong Y, Roos M, Gruijters T, Donaldson P, Bullivant S, Beyer E, Kistler J. Differential expression of two gap junction proteins in corneal epithelium. Eur J Cell Biol. 1994;64(1): 95–100.

31. Wolosin JM, Xiong X, Schütte M, Stegman Z, Tieng A. Stem cells and differentiation stages in the limbo-corneal epithelium. Prog Retin Eye Res. 2000;19(2):223–255.
32. Igarashi T, Shimmura S, Yoshida S, Tonogi M, Shinozaki N, Yamane G. Isolation of oral epithelial progenitors using collagen IV. Oral Dis. 2008;14(5):413–418.
33. Chee KY, Kicic A, Wiffen SJ. Limbal stem cells: the search for a marker. Clin Expl Ophthalmol. 2006;34(1):64–73.
34. Moharamzadeh K, Brook I, Van Noort R, Scutt A, Thornhill M. Tissue-engineered oral mucosa: a review of the scientific literature. J Dent Res. 2007;86(2):115–124.
35. Lan L, Luo Y, Cui D, Shi B-Y, Deng W, Huo L-L, Chen H-L, Zhang G-Y, Deng L-L. Epithelial-mesenchymal transition triggers cancer stem cell generation in human thyroid cancer cells. Int J Oncol. 2013;43(1):113–120.
36. Cervenkova K, Belejova M, Vesely J, Chmela Z, Rypka M, Ulrichová J, Modriansky M, Maurel P. Cell suspensions, cell cultures, and tissue slices-important metabolic in vitro systems. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2001;145(2):57–60.
37. Li D-Q, Chen Z, Song XJ, de Paiva CS, Kim H-S, Pflugfelder SC. Partial enrichment of a population of human limbal epithelial cells with putative stem cell properties based on collagen type IV adhesiveness. Exp Eye Res. 2005;80:581–590.
38. Pellegrini G, Dellambra E, Golisano O, Martinelli E, Fantozzi I, Bondanza S, Ponzin D, McKeon F, De Luca M. p63 identifies keratinocyte stem cells. Proc Natl Acad Sci U S A. 2001; 98(6):3156–3161.
39. Ramachandran C, Basu S, Sangwan VS, Balasubramanian D. Concise review: the coming of age of stem cell treatment for corneal surface damage. Stem Cells Transl Med. 2014;3(10): 1160–1168.
40. Dobrowolski D, Orzechowska-Wylegala B, Wowra B, Wroblewska-Czajka E, Grolik M, Szczubialka K, Nowakowska M, Puzzolo D, Wylegala EA, Micali A. Cultivated oral mucosa epithelium in ocular surface reconstruction in aniridia patients. Biomed Res Int. 2015;2015:1–7.
41. Li W, Li Q, Wang W, Li K, Ling S, Yang Y, Liang L. A rat model of autologous oral mucosal epithelial transplantation for corneal limbal stem cell failure. Eye Sci. 2014;29(1):1–5.
42. Ilmarinen T, Laine J, Juuti-Uusitalo K, Numminen J, Seppänen-Suuronen R, Uusitalo H, Skottman H. Towards a defined, serum-and feeder-free culture of stratified human oral mucosal epithelium for ocular surface reconstruction. Acta Ophthalmol. 2013;91(8):744–750.
43. Murakami D, Yamato M, Nishida K, Ohki T, Takagi R, Yang J, Namiki H, Okano T. Fabrication of transplantable human oral mucosal epithelial cell sheets using temperature-responsive culture inserts without feeder layer cells. J Artif Organs. 2006;9(3):185–191.
44. Kim IL, Khetan S, Baker BM, Chen CS, Burdick JA. Fibrous hyaluronic acid hydrogels that direct MSC chondrogenesis through mechanical and adhesive cues. Biomaterials. 2013; 34(22):5571–5580.
45. Rodríguez-Seguí SA, Pla-Roca M, Engel E, Planell JA, Martínez E, Samitier J. Influence of fabrication parameters in cellular microarrays for stem cell studies. J Mater Sci Mater Med. 2009;20:1525–1533.
46. Cotsarelis G, Cheng S-Z, Dong G, Sun T-T, Lavker RM. Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. Cell. 1989;57(2):201–209.
47. Sun T-T, Tseng SC, Lavker RM. Location of corneal epithelial stem cells. Nature. 2010;463: E10–E11.
48. Ozturk SS, Palsson BØ. Effect of initial cell density on hybridoma growth, metabolism, and monoclonal antibody production. J Biotechnol. 1990;16:259–278.
49. Takagi M, Umetsu Y, Fujiwara M, Wakitani S. High inoculation cell density could accelerate the differentiation of human bone marrow mesenchymal stem cells to chondrocyte cells. J Biosci Bioeng. 2007;103(1):98–100.
50. Li J, Jones B, Zhang Y, Vinardell T, Pei M. Low-density expansion protects human synovium-derived stem cells from replicative senescence: a preliminary study. Drug Deliv Transl Res. 2012;2(5):363–374.
51. Mitsutake N, Iwao A, Nagai K, Namba H, Ohtsuru A, Saenko V, Yamashita S. Characterization of side population in thyroid cancer cell lines: cancer stem-like cells are enriched partly but not exclusively. Endocrinology. 2007;148:1797–1803.
52. Turner JG, Gump JL, Zhang C, Cook JM, Marchion D, Hazlehurst L, Munster P, Schell MJ, Dalton WS, Sullivan DM. ABCG2 expression, function, and promoter methylation in human multiple myeloma. Blood. 2006;108(12):3881–3889.
53. Matic M, Evans WH, Brink PR, Simon M. Epidermal stem cells do not communicate through gap junctions. J Invest Dermatol. 2002;118:110–116.
54. Mennecier G, Derangeon M, Coronas V, Hervé JC, Mesnil M. Aberrant expression and localization of connexin43 and connexin30 in a rat glioma cell line. Mol Carcinog. 2008;47(5): 391–401.
55. Jiang JX, Gu S. Gap junction-and hemichannel-independent actions of connexins. Biochim biophys acta. 2005;1711(2): 208–214.
56. Rodriguez-Jimenez FJ, Alastrue-Agudo A, Stojkovic M, Erceg S, Moreno-Manzano V. Connexin 50 expression in ependymal stem progenitor cells after spinal cord injury activation. Int J Mol Sci. 2015;16(11):26608–26618.