Human Conjunctival Stem Cells are Predominantly Located in the Medial Canthal and Inferior Forniceal Areas

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Purpose. The conjunctiva plays a key role in ocular surface defence and maintenance of the tear film. Ex vivo expansion of conjunctival epithelial cells offers potential to reconstruct the ocular surface in cases of severe cicatrising disease, but requires initial biopsies rich in stem cells to ensure long-term success. The distribution of human conjunctival stem cells, however, has not been clearly elucidated.

Methods. Whole human cadaveric conjunctiva was retrieved and divided into specific areas for comparison. From each donor, all areas from one specimen were cultured for colony-forming efficiency assays and immunocytochemical studies; all areas from the other specimen were fixed and paraffin embedded for immunohistochemical studies. Expression of CK19, p63, and stem cell markers ABCG2, ΔNp63, and Hsp70 were analyzed. Results were correlated to donor age and postmortem retrieval time.

Results. Conjunctiva was retrieved from 13 donors (26 specimens). Colony-forming efficiency and expression of stem cell markers ABCG2, ΔNp63, and Hsp70 in cultures and ABCG2 in fixed tissue were all consistently demonstrated throughout the tissue but with highest levels in the medial canthal and inferior forniceal areas ($P < 0.01$ for each). Both increasing donor age and longer postmortem retrieval times were associated with significantly lower colony-forming efficiency, stem cell marker expression in cell cultures and ABCG2 expression in fixed tissue.

Conclusions. Biopsies from the medial canthus and inferior forniceal areas, from younger donors, and with short postmortem retrieval times offer the greatest potential to developing conjunctival stem cell–rich epithelial constructs for transplantation.

Keywords: conjunctiva, stem cell, progenitor cell, medial canthus, inferior fornix

The conjunctiva is a mucous membrane that forms the majority of the ocular surface. It is of integral importance: to provide immunological defence, and to sustain a healthy tear film, which in turn prevents ocular infection and desiccation of the corneal epithelium, thus preserving vision.[1] The conjunctiva is susceptible to a wide spectrum of diseases, including trachoma, chemical and thermal burns, mucous membrane pemphigoid and Stevens-Johnson syndrome, which may result in cicatrisation, chronically painful eyes and blindness.[2,3] Treatment modalities for these severe inflammatory disorders are presently very limited and primarily aimed at alleviation of symptoms and prevention of disease progression.

The identification of corneal stem cells at the limbus,[4–12] has led to the development of transplantation of ex vivo expanded limbal epithelium as a therapy for limbal stem cell deficiency.[13] Significant clinical improvements in corneal clarity and ocular surface stability have been achieved with these methods.[14–18] Reported success rates however, vary greatly,[19] which may in part may be attributable to coexisting severe conjunctival disease.

The ability to similarly ex vivo expand and transplant conjunctival stem cells may address these and other causes of conjunctival insufficiency, such as postexcision of extensive conjunctival neoplasms. To achieve this goal the conjunctival stem cells must first be localized and characterized.[20]

Research indicates that the corneal and conjunctival epithelia are of distinct cell lineages.[21–23] Whereas there is substantial evidence of the corneal stem cells residing at the limbus,[4–12] the source of conjunctival stem cells has been debated. Animal studies have predominantly been label retention studies and have generated much conflicting evidence.[24–33] There have been very few studies of human conjunctiva, with no complete assessment of the tissue as a whole.[10,22,34–36] This study was thus conducted as the first comprehensive assessment (to determine both clonogenic ability and expression of stem cell markers) across the whole human conjunctival tissue, in order to ascertain the location of conjunctival stem cells.

Materials and Methods

Human Conjunctival Tissue

Ethical approval was obtained, and the study performed in accordance to the standards of the 1964 Declaration of Helsinki. Informed consent was obtained from donor relatives

prior to their inclusion in the study. Whole human conjunctival tissue was obtained from donors at The Royal Liverpool University Hospital (Liverpool, UK) within 28 hours of death, using an established surgical technique.[37] In addition to the standard exclusion criteria for ocular tissue donation,[38] an additional exclusion of those who had undergone previous eyelid surgery was applied. Tissue was retrieved from all such donors available over a 3-year period.

Conjunctival tissue was retrieved from both eyes from each donor by a single surgeon (RS). Each right conjunctiva was processed for cell culture studies and the left fixed and paraffin-embedded for immunohistochemical staining. Data on donor age, sex, cause of death, and postmortem retrieval time was recorded for comparative analysis.

Epithelial Cultures

Materials throughout were from Sigma-Aldrich (Gillingham, UK) unless otherwise stated. Whole conjunctival specimens were washed in PBS containing 2% penicillin-streptomycin and 2% fungizone for 1 hour at room temperature with gentle agitation. The underlying fat and connective tissue was removed using sterile Moorfields dissecting forceps and Westcott scissors (Malosa Medical, Halifax, UK), taking care not to damage the epithelium. The conjunctival epithelium was surgically divided using Westcott scissors into eight approximately equal areas representing the tarsal, forniceal, and bulbar areas of both superior and inferior conjunctiva, and the medial and lateral canthal areas, as shown in a schematic diagram in Figure 1A. The medial and lateral canthal areas comprised the closely connected bulbar, forniceal, and tarsal tissue immediately adjacent to the canthi, and were hence termed canthal areas for ease of nomenclature.

Each area of conjunctival epithelium was finely chopped with scissors and incubated with 1 mL 0.05% trypsin 0.02% EDTA at 37°C for 20 minutes with gentle agitation. The trypsin-cell solution was removed and neutralized in 10 mL conjunctival epithelial media (described below). This cycle was repeated a further three times with fresh trypsin added to the tissue each time. The cell suspensions were subjected to repeat pipetting to disperse the cell clumps and passed through a 70-μm pore cell strainer (BD Biosciences, Oxford, UK), before resuspending in media.

Murine J23T3 fibroblasts were cultured in high glucose (4.5 g/L) Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Paisley, UK) with 10% fetal calf serum (FCS), 1% penicillin-streptomycin and 1% fungizone until confluency, whereon they were trypsinized and resuspended to 1×10^7 cells/mL, and treated with mitomycin C (20 μg/mL) at 37°C for 1 hour with gentle agitation to prepare a feeder layer of 1×10^5 cells/cm^2.

Primary conjunctival cells at passage zero were seeded at 15,000 cells/cm^2 onto the 3T3 feeder layer, and maintained with conjunctival epithelial cell media: three parts low glucose (1 g/L) DMEM (Invitrogen) and one part Ham's F12 media (Invitrogen), containing 10% FCS, 1% penicillin-streptomycin, 1% fungizone, 5 μg/mL transferrin, 5 μg/mL insulin, 1.4 ng/mL triiodothyronine, 12 μg/mL adenine, 0.4 μg/mL hydrocortisone, and 0.1 μg/mL epidermal growth factor. Cells were cultured for 10 to 14 days at 37°C with 5% CO_2 to confluence.

Colony-Forming Efficiency (CFE) Assays

To evaluate the clonogenic ability of the cells, CFE assays were performed at passage 1. Viable conjunctival cells (as assessed with 0.4% trypan blue) from each conjunctival area were seeded at 500, 1000, and 2000 cells per 9.6-cm^2 well (Greiner, Stonehouse, UK), onto a mitomycin C–treated J23T3 feeder layer. On the 16th day of culture, cells were fixed with 3.7% neutral-buffered formaldehyde (NBF; Bios Europe Limited, Skelmersdale, UK) for 10 minutes, washed with PBS, and stained with 1% rhodamine B (Sigma-Aldrich) in methanol (Fisher Scientific, Loughborough, UK) for 10 minutes at room temperature. Colonies per well were counted and using the formula: number of colonies formed/number of cells plated × 100. The CFE (%) was calculated as the mean value across the three wells.

Immunofluorescent Staining

Immunofluorescent staining was performed at passage 1, at subculturing onto fresh 3T3 feeder layers at 6000 cells/cm^2 in eight-chamber slides (VWR, Lutterworth, UK). Cells were fixed at day 9 with 100% methanol (Fisher Scientific) at −20°C for 10 minutes. After permeabilization and blocking together with 0.1% tween, 1% Bovine Serum Albumin and 10% goat serum for 1 hour, primary antibodies against CK19 (1:50, clone RCK108; Dako, Ely, UK), ABCG2 (1:20, clone 21; Millipore, Watford, UK), p63 (1:100, clone ΔN; Biolegend, London, UK), and Hsp70 (1:1000, Clone BRM-22; Abcam, Cambridge, UK) were applied and incubated for 1 hour at room temperature. Following PBS washes, a secondary antibody, Alexafluor 488 goat-anti-mouse/anti-rabbit IgG (Invitrogen) was applied 1:250 in PBS for 1 hour, followed by PBS washes and counterstaining with 1% propidium iodide (PI) in 10% RNase for 10 minutes. Slides were mounted with aqueous fluorescent mountant (Dako) and imaged on a Polyvar microscope (Reichert Jung, Buffalo, NY, USA). Human limbal cell cultures were used as positive controls and 1% goat serum alone was used as a negative control. Expression of immunocytochemical markers was graded quantitatively by the number of positively staining cells per average of five ×20 magnification random fields of view with a grading scale of 0 cells: −, less than five cells: ±, 5 to 10 cells: +, 10 to 15 cells: ++, 15 to 20 cells: +++, and greater than 20 cells: ++++.

Tissue Fixation, Embedding, and Sectioning

Whole conjunctival specimens were similarly prepared and dissected into 10 areas: Both superiorly and inferiorly, the lids were divided into medial, central, and lateral sections encompassing complete tissue from the bulbar conjunctiva adjacent to the limbus to that at the eyelid margin as previously described.[37] The other sections comprised the superior and inferior zones of the canthal areas as previously described. Each section was pinned onto cork board and fixed with 3.7% NBF for 24 hours. The specimens were processed through ethanol (Department of Chemistry, University of Liverpool, Liverpool, UK), xylene (Fisher Scientific), and Formula R paraffin wax (Surgipath, Milton Keynes, UK) washes overnight using a tissue processor (Shandon, Runcorn, Cheshire, UK) and embedded in paraffin using an embedding unit (Shandon). Four-micrometer sections were cut using a microtome (Shandon) in the axial plane from the canthal specimens and in the sagittal plane from all other specimens, and wet mounted onto X-tra adhesive slides (Surgipath).

Immunohistochemical Staining

Slides were deparaffinized with a series of 5-minute xylene and 100% then 70% ethanol washes. Prior to ABCG2 staining, antigen retrieval was first achieved by incubating in 0.01 M citrate (BDH, Bristol, UK) buffer pH6 for 10 minutes at 95°C. Slides were rinsed in 0.05% PBS tween before and after blocking of endogenous peroxidases with 0.03% hydrogen peroxide (Envision kit; Dako) for 10 minutes, and nonspecific blocking with 20% goat serum for 30 minutes. Following incubation with primary antibodies to ABCG2 (1:20, clone 21; Millipore) and

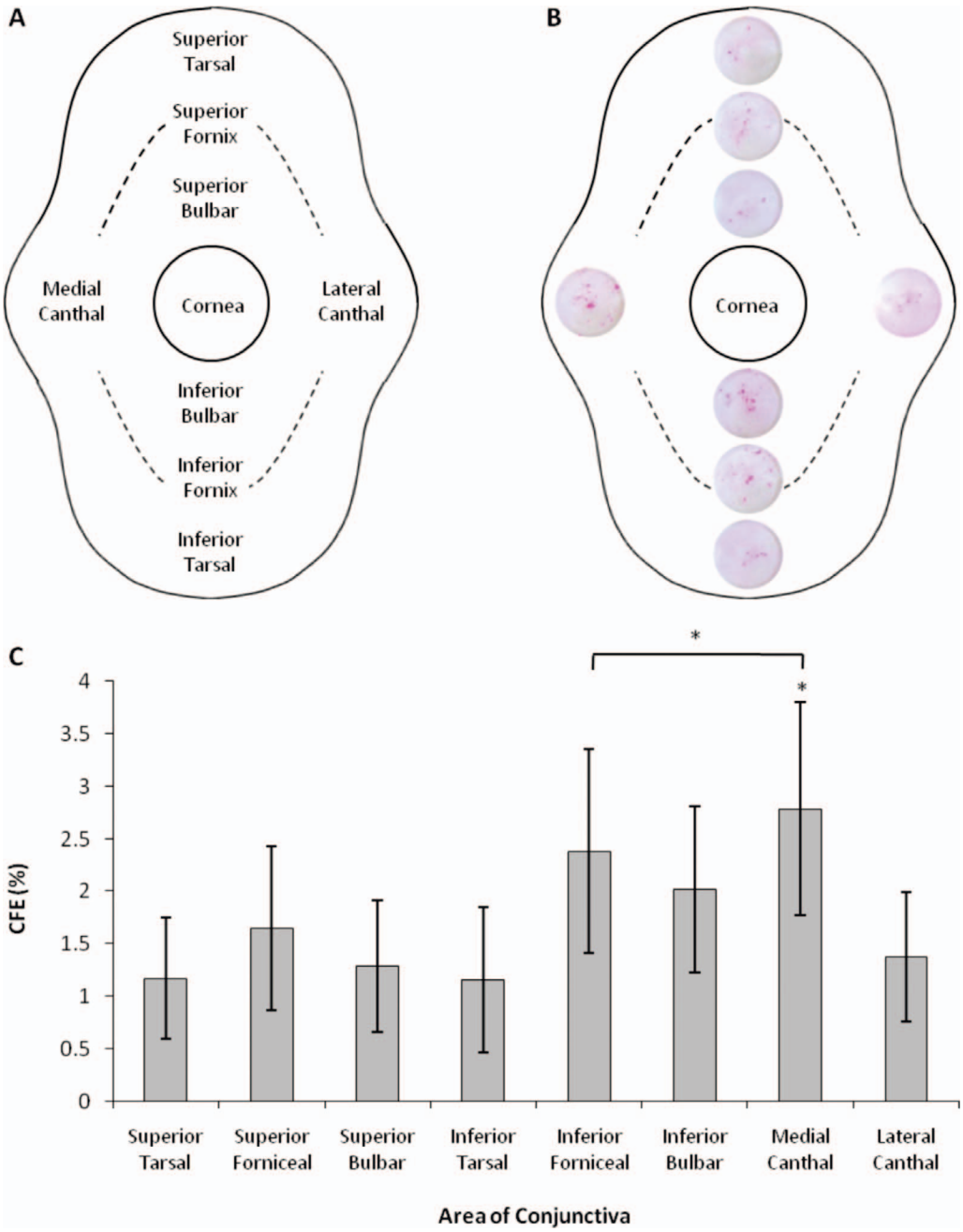

Figure 1. (**A**) Schematic diagram of the whole conjunctiva with fornices represented by *dashed lines* and eight areas assessed for CFE analysis labeled. (**B**) Colony-forming efficiency images from one donor across all areas of the conjunctiva. (**C**) Histogram demonstrating the mean overall CFE values across all areas of the conjunctiva from all donors. The highest CFE was observed in the medial canthal area alone (*$P < 0.01$), and medial canthal and inferior forniceal areas combined (*$P < 0.01$). *Error bars* ±1 SD.

p63 (1:50, clone 4A4; Dako) for 2 hours at room temperature, slides were washed with 1% goat serum before incubating with the secondary antibody, horseradish peroxidase anti-mouse (Envision kit) for 30 minutes. An AEC chromagen (Envision kit) was allowed to develop for 10 to 15 minutes, and after counterstaining with haematoxylin mayer (Surgipath) for 30 seconds, the slides were mounted with Aquatex aqueous mountant (Merck, Hoddeston, UK) and imaged on an Olympus BX60 microscope (Olympus Corporation, Tokyo, Japan). Placental and limbal tissue was used as positive controls and 1% goat serum alone was used as a negative control.

A representative image was taken from the central area of each tarsal, forniceal, and bulbar zone, along each superior and inferior specimen, and from the central area of each canthal specimen, hence giving 22 comparative areas across the whole tissue (see schematic diagram in Fig. 3). Expression of immunohistochemical markers in tissue sections was graded quantitatively by the proportion of positively staining epithelial cells throughout the epithelium: 0 cells: −, less than or equal to 1/3 cells: +, 1/3 to 2/3 cells: ++, and greater than or equal to 2/3 cells: +++.

Statistical Analysis

The variability of CFE immunocytochemical and immunohistochemical staining across different areas of the conjunctiva

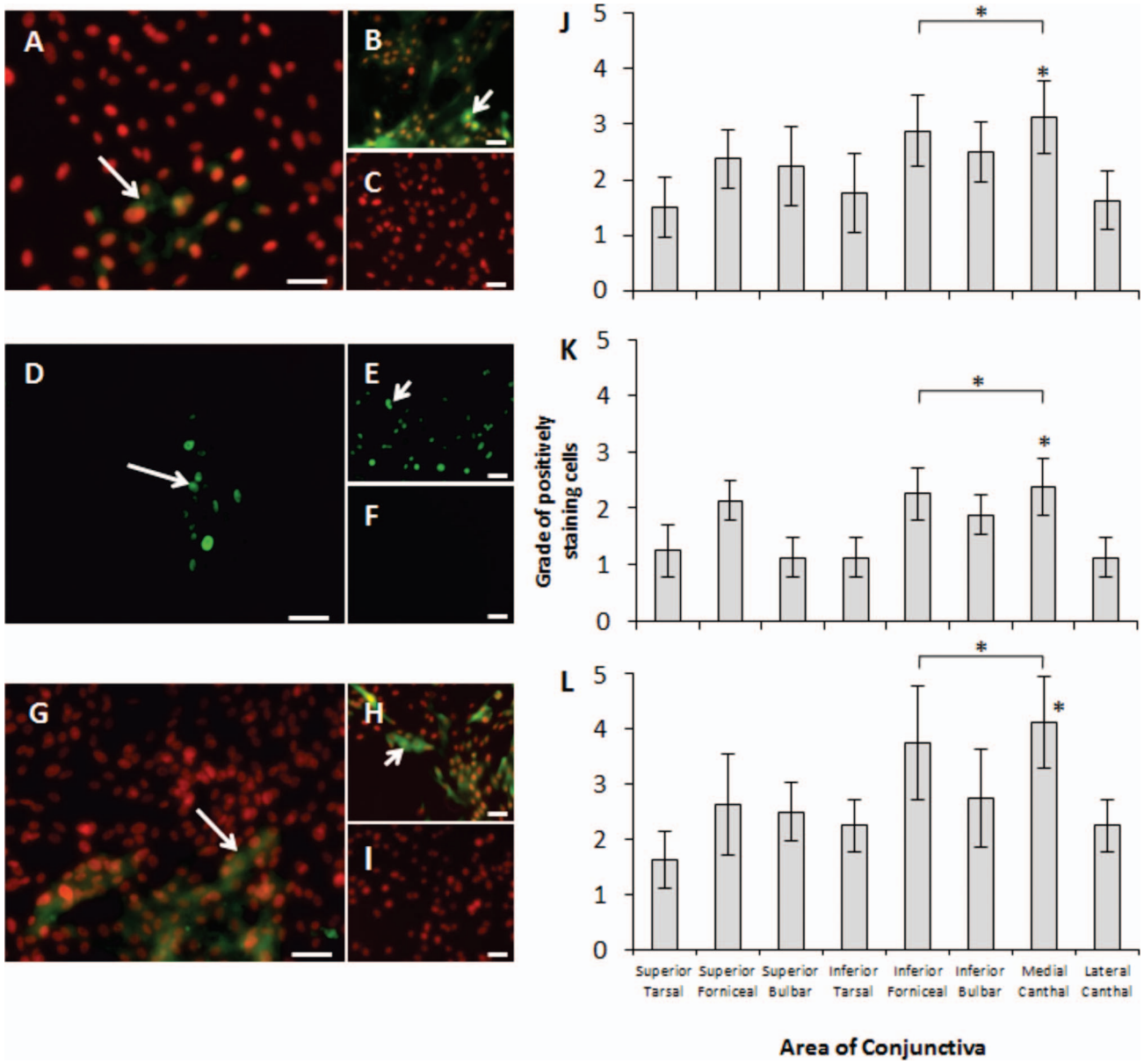

Figure 2. (**A–C**) Photomicrographs of immunofluorescent staining for ABCG2 (*arrows*), with human limbal positive control (**B**) and negative control (**C**). (**D–F**) Photomicrographs of immunofluorescent staining for ΔNp63 (*arrows*), with human limbal positive control (**E**) and negative control (**F**). (**G–I**) Photomicrographs of immunofluorescent staining for Hsp70 (*arrows*), with human limbal positive control (**H**) and negative control (**I**). *Scale bars*: 50 μm. (**J**, **K**) Histograms demonstrating the mean overall staining across all areas of the conjunctiva from all donors for ABCG2 (**J**), ΔNp63 (**K**), and Hsp70 (**L**). Highest grades of staining were observed in the medial canthal (*$P < 0.01$), and together with inferior forniceal areas (*$P < 0.01$). *Error bars* ±1 SD.

was assessed with a Friedman test, and post hoc analysis with a Wilcoxon rank sum test. Bonferroni corrections were made to account for multiple tests. The correlation between immunofluorescent staining for different markers and between these, CFE and immunohistochemical staining were each assessed with a Kendall's Tau correlation coefficient. The effect and interaction of donor age and postmortem retrieval time on CFE and immunohistochemical staining was assessed with a linear mixed-effect model, with fixed effect of area, age, and postmortem retrieval time. Random intercept was used to account for correlations between measurements from the same donor. All of these tests were performed using SPSS Statistics 20 (SPSS, Inc., Chicago, IL, USA). P less than or equal to 0.05 was considered statistically significant ($\alpha = 5\%$).

Results

Conjunctiva was retrieved from 13 donors in total, giving 26 whole conjunctival specimens. These included 5 male and 13 female donors, aged 22 to 92 (mean 74, median 82) years; all of which were Caucasian. The postmortem retrieval times varied from 8 to 28 (mean 20.4, median 21.5) hours.

Cultures From The Medial Canthal and Inferior Forniceal Areas Have the Highest CFE

Colony-forming efficiency assays were compared across eight different areas of the conjunctiva (Fig. 1A), from eight donors. This demonstrated significant overall variation across the tissue as a whole, with a consistent pattern shown in each donor (P

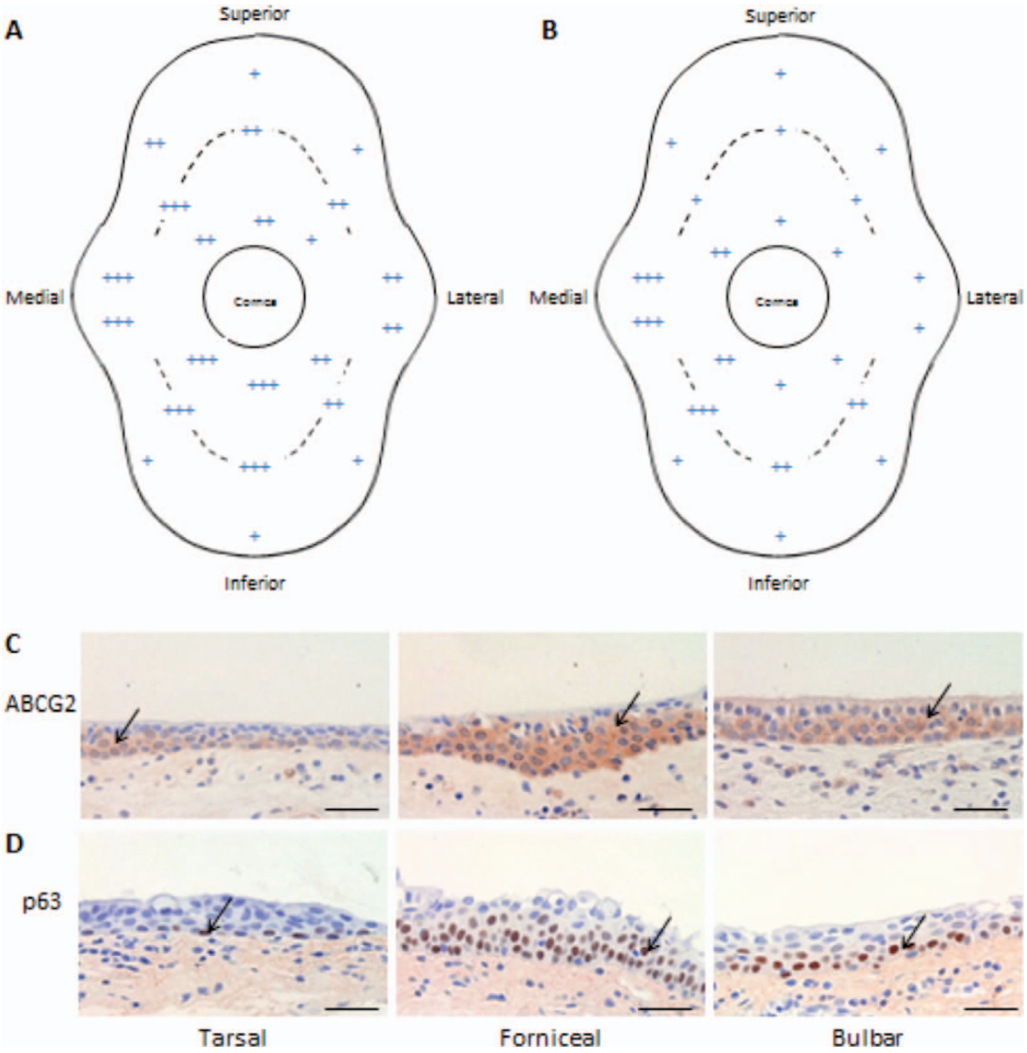

FIGURE 3. (**A**, **B**) Schematic diagrams of the whole conjunctiva with fornices represented by *dashed lines*, demonstrating average grades of immunohistochemical reactivity for ABCG2 (**A**) and p63 (**B**). Highest grades of staining are observed in the medial canthal, inferior medial, and inferior central forniceal areas ($P < 0.01$). (**C**, **D**) Representative photomicrographs of immunohistochemical staining for ABCG2 (**C**) and p63 (**D**) across the inferior tarsal, forniceal, and bulbar conjunctiva. Positive immunoreactivity is shown in *brown* (*arrows*) with haematoxylin counterstaining in *blue*. Mild immunoreactivity is observed in the tarsal area, intense in the forniceal area and moderate staining in the bulbar area. *Scale bars*: 50 μm.

< 0.01). An example of images from one donor is demonstrated in Figure 1B. Overall highest CFE levels were consistently noted in the medial canthal area alone ($P < 0.01$), and in the medial canthal and inferior forniceal areas together ($P < 0.01$) compared with all other areas. Mean data from all eight donors is demonstrated in Figure 1C. Higher CFE was shown in the fornices than bulbar than tarsal areas ($P < 0.01$), and this pattern was replicated in both superior and inferior conjunctiva, with higher CFE in inferior compared with superior areas ($P < 0.01$).

Cultures From the Medial Canthal and Inferior Forniceal Areas Have the Highest Levels of Stem Cell Marker Expression

Intense expression of CK19 was demonstrated consistently across all cultures used for immunofluorescent staining, confirming the presence of conjunctival epithelial cells in culture. Immunofluorescent staining with ABCG2, ΔNp63, and Hsp70 antibodies (Figs. 2A–I) were assessed from all eight areas of the conjunctiva from the same eight donor tissues, which were used for CFE analysis.

Staining patterns were consistently demonstrated across the different anatomical areas for each donor tissue, with significant variation across the tissue as a whole for each marker ($P < 0.01$ for each). Highest levels of expression were demonstrated in the medial canthal and forniceal areas, especially inferiorly for each. Statistical significance was noted to both the higher level of expression in the medial canthal area alone ($P < 0.01$ for each), and in the medial canthal and inferior forniceal areas grouped together ($P < 0.01$ for each). Mean values across each area for each stain are demonstrated in Figures 2J through L. Lowest levels of expression were demonstrated in the tarsal conjunctival epithelium, hence the pattern of increased expression in the forniceal than bulbar than tarsal areas was again apparent for each stain. Levels of expression were not however significantly higher in the inferior compared to superior areas for either ABCG2 ($P = 0.29$), ΔNp63 ($P = 0.14$), or Hsp70 ($P = 0.18$).

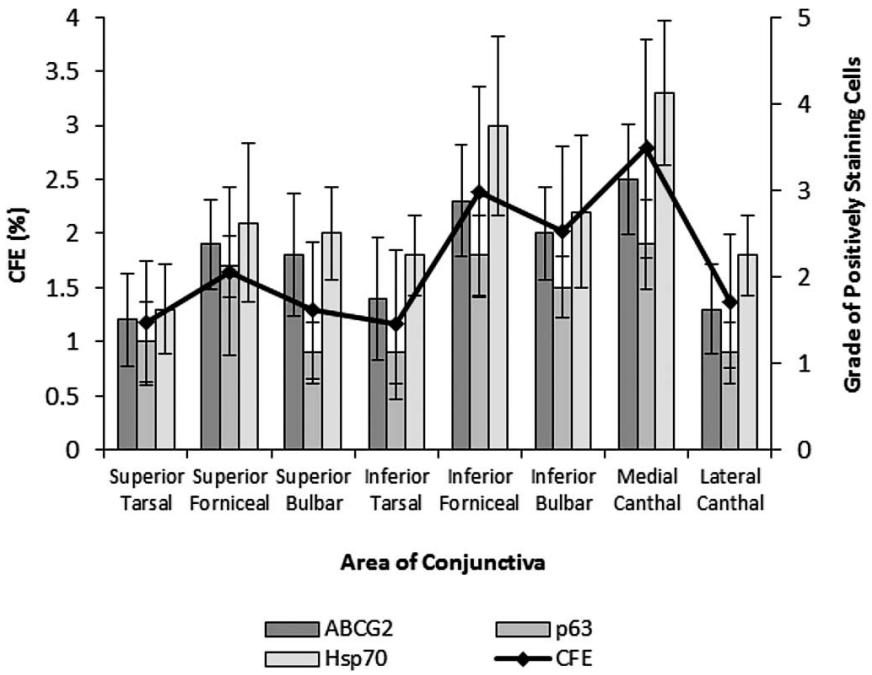

FIGURE 4. Graph demonstrating both the mean overall CFE and immunofluorescent expression of stem cell markers in cell cultures from all donors across the different areas of the conjunctiva. Correlations were demonstrated between each stain independently ($P < 0.01$ for each) and for each stain to CFE independently ($P < 0.01$ for each). *Error bars* ±1 SD.

Tissue Sections From the Medial Canthal and Inferior Forniceal Areas Have the Highest Levels of Stem Cell and Transit-Amplifying Cell Marker Expression

ABCG2 expression was demonstrated across all areas of the conjunctiva in the basal layers of the epithelium. Staining across sections from 10 donors was compared. Significant variation in expression was demonstrated across the whole tissue ($P < 0.01$). The overall gradings averaged from all donors are demonstrated on a schematic diagram in Figure 3A. Highest levels of staining (grade +++) were demonstrated in a region from the medial canthal to inferior forniceal areas ($P < 0.01$). In these areas staining was demonstrated in the majority of epithelial cells, but most intense basally, with often only the superficial epithelial layers spared. Lowest levels of staining were demonstrated in the tarsal conjunctival epithelium, hence the same pattern of more intense staining in the forniceal than bulbar than tarsal areas was demonstrated again (Fig. 3C). This was replicated in both superior and inferior conjunctiva, with highest levels inferiorly ($P < 0.01$). These patterns were consistently demonstrated across each donor tissue.

p63 expression was similarly demonstrated across all areas of the conjunctiva in the basal layers of the epithelium. Significant variation in staining was demonstrated across the whole tissue ($P = 0.02$). The overall averaged gradings are demonstrated on a schematic diagram in Figure 3B. The distribution across the tissue, although of a similar pattern to that seen for ABCG2, was not significant. In the medial canthal and inferior forniceal areas staining was additionally demonstrated in the intermediate layer of the epithelium. The patterns of greater expression in the fornices than bulbar than tarsal areas (Fig. 3D), and greater expression in the inferior than superior conjunctiva were also replicated, but not significantly ($P = 0.07$).

Although the areas of most intense ABCG2 and p63 expression within the conjunctival epithelium are also rich in goblet cells,[39] no correlation was noted between direct proximity of ABCG2 or p63 positively staining cells to goblet cells. Indeed varying intensity of staining to both markers was observed in close proximity to clusters of multiple goblet cells, with no clear pattern to different areas of the tissue.

Significant Correlations Were Made Between Immunofluorescent Staining for Each Stem Cell Marker, CFE, and Immunohistochemical Staining for ABCG2

Significant correlations were shown between the distribution patterns of immunofluorescent staining for each stem cell marker in cultured cells independently ($P < 0.01$ for each), and of each marker to CFE ($P < 0.01$ for each). This data is summarized in Figure 4. Similarly (including data from only five donors, which were used in both assessments), significant associations were noted between immunohistochemical staining for ABCG2 and CFE ($P < 0.01$), and between immunohistochemical staining for ABCG2 and immunofluorescent staining for each stem cell marker independently ($P < 0.01$ for each).

Increasing Donor Age is Associated With a Reduction in CFE, Immunofluorescent, and Immunohistochemical Stem Cell Marker Expression

Whole conjunctival CFE was determined as the average CFE reading from all eight individual areas for each donor and was compared with donor age. Although there was a significant clustering of older donors, there was a significant reduction of CFE with increasing donor age ($P < 0.01$). Whole conjunctival immunofluorescent and immunohistochemical staining were similarly determined by the average values across all areas of

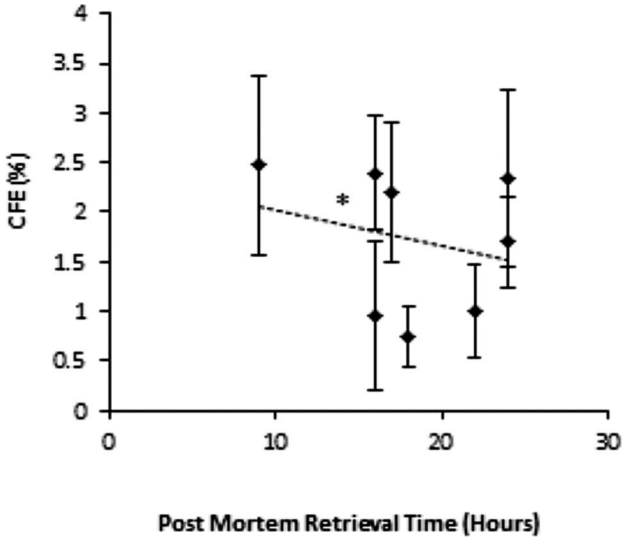

FIGURE 5. Line graph demonstrating the variance in whole conjunctival CFE with postmortem retrieval time. A decrease in CFE value was demonstrated (*$P < 0.01$). *Error bars* ±1 SD.

the tissues. Increasing donor age was significantly associated with lower immunofluorescent staining for ABCG2 and Hsp70, and immunohistochemical staining for ABCG2 ($P < 0.01$ for each). No relationship, however, was evident between immunofluorescent staining for ΔNp63 and donor age ($P > 0.1$).

Increasing Postmortem Retrieval Time is Associated With a Reduction in CFE, and Some Immunofluorescent and Immunohistochemical Stem Cell Marker Expression

Similarly, whole CFE and stem cell marker staining for each donor was compared with postmortem retrieval time. Longer postmortem retrieval times were associated with significantly lower CFE ($P < 0.01$; Fig. 5), and lower immunofluorescent and immunohistochemical staining for ABCG2 ($P < 0.01$ for each). No relationship was evident between immunofluorescent staining for ΔNp63 ($P = 0.45$) or Hsp70 ($P > 0.1$) and donor age.

DISCUSSION

Substantial evidence from each aspect of this study (Figs. 1–4) indicates the presence of stem cells throughout the human conjunctival epithelium, but with significantly highest concentrations in the medial canthal and inferior forniceal areas. The significant positive correlations between clonogenic ability and stem cell marker expression in both cultured cells and fixed tissue adds further credence to these conclusions. These findings are in keeping with other reports suggesting that human conjunctival epithelial stem cells are located predominantly in the fornix,[10,35] but are also present in other areas.[10,22,34,35,40]

Only one previous study has assessed the clonogenic ability across the human conjunctival epithelium.[22] Although in contrast the authors noted comparable rates across four bulbar and two forniceal areas, assays were only compared from cells obtained from single 1- to 2-mm^2 biopsies from a single donor, and did not include assessment of the tarsal regions. If indeed stem cells are scattered throughout the epithelium such as is proposed in the skin,[41] assuming their distribution is not uniform, this latter method risks sampling areas with inaccurate representations of stem cell concentration. Although some studies have examined the expression of a more extensive array of stem cell markers,[34,35,40] similarly, none of these studies examined the whole tissue. More so, this present study assesses tissue from a greater number of donors than any of the aforementioned studies, and attempts to localize the stem cells by both clonogenic ability and stem cell marker expression in both fixed tissue and cultured cells from the same donors.

Immunohistochemical studies demonstrated widespread ABCG2 and p63 expression in the basal layers of the epithelium (Fig. 3), a finding confirmed in areas of the conjunctiva in other studies.[10,34,40] p63 expression was assessed as no antibody to ΔNp63 for immunohistochemistry was available. No distinct defined collections of stem cells or transit amplifying cells, and no obvious morphologically distinct region or niche for the conjunctival stem cell was noted, such as are seen in the limbus as the source of corneal epithelial cells[11,42,43] It is therefore plausible that there are pockets of stem cells throughout the basal layer of the human conjunctival epithelium[40] and that a similar renewal model to the interfollicular stem cell model proposed in the skin,[41] is also true of the conjunctival epithelium.

As the richest source of human conjunctival stem cells, the medial canthal and inferior forniceal areas may offer greater physical protection to the stem cell niches. But perhaps more importantly, the overall distribution of conjunctival stem cells demonstrated in this study mimics that of the human goblet cells and intraepithelial mucous crypts.[39] Indeed, more intense ABCG2 staining has been previously correlated to the goblet cell–rich areas of the conjunctiva.[10] There was not however, an apparent direct relationship in immediate colocalization of these cells within the epithelium in immunohistochemical studies; with very variable levels of both ABCG2 and p63-positive cells noted adjacent to clusters of goblet cells (data not shown). Similarly, the crypt stem cells in the colonic epithelium are not noted in immediate proximity to goblet cells.[44] This distribution pattern may simply reflect the pattern of epithelial renewal in the conjunctival epithelium: As goblet cells arise from a common conjunctival bipotent stem or transit amplifying cell,[22] thus greater concentrations would perhaps be expected in proximity to them. However, their coexistence may alternatively be essential to the conjunctival stem cell niche; and it is perhaps not coincidental that the bulge stem cells of the skin epidermal hair follicle also reside in close proximity to sebaceous glands.[41,45]

The medial canthal and inferior forniceal areas, and in particular the plica semilunaris and caruncle, are also densely vascularized and contain melanocytes,[46,47] and the plica semilunaris is abundantly infiltrated with both specific and nonspecific immune cells.[47] These features are common to other stem niches such as the limbus.[48] Rich vascularization provides a plentiful supply of nutrients, blood-borne growth and survival factors.[49] Melanin offers protection against the carcinogenic insult from ultraviolet light and subsequent formation of reactive oxygen species.[50] It is interesting to note that whereas the limbal stem cells are predominantly located superiorly and inferiorly,[42] the conjunctival stem cells reside predominantly close to the midline. It has been suggested that the plica semilunaris plays an important role as a specialized organ in ocular defence,[47] but perhaps its significance extends further than this, to also incorporate conjunctival epithelial renewal.

Stem cell niche microenvironments of a variety of tissues are reported to deteriorate with age, with a subsequent critical effect on stem cell number, phenotype, and clonogenic ability.[51–54] Indeed, increasing donor age was associated with

significantly lower clonogenic efficiency, and expression of stem cell markers in cultured cells and tissue sections in this study. A previous study, however, reported no significant difference in conjunctival cell proliferation with donor age.[55] In terms of limbal cell cultures, variable associations have been noted between CFE, culture success rates, and proliferative potential with increasing donor age,[56–58] but no relationship to ABCG2 or ΔNp63α levels.[58] Significant associations were also demonstrated in this study between increasing postmortem retrieval time and lower clonogenic ability (Fig. 5), and lower expression of ABCG2 in both tissue sections and cultured cells. It is interesting to note, that although significant correlations between ABCG2 expression and both increasing donor age and postmortem retrieval time, and between Hsp70 expression and increasing donor age were detected, no correlation was detected between ΔNp63 expression and either factor. Although p63 is considered a transit amplifying cell marker,[59] the ΔNp63 antibody encompasses the ΔNp63α isoform, which is considered a relatively pure stem cell marker.[12] The reduction of CFE is perhaps however more clinically significant.[58]

Knowledge of the distribution of conjunctival stem cells ensures biopsies taken for ex vivo expansion for epithelial replacement can be taken from stem cell–rich areas to provide optimal transplantation success.[42,60,61] Furthermore, for those patients with particularly severe disease who are unable to provide an adequate biopsy, the significance of younger donor age and shorter retrieval times to increasing stem cell yield and clonogenic ability in culture will enable optimal selection of allograft donor tissue. But the implications also extend beyond this: these stem cell–rich areas should be avoided or damage to them minimized during conjunctival or other ocular surgery. Additionally, the medial canthal and inferior forniceal areas are the sites where any topically administered eye medications will naturally accumulate, and are thus most exposed to toxicity from the preservatives within. It is therefore perhaps imperative that preservative-free topical ophthalmic medications are considered in any patient with significant conjunctival disease. It is also noteworthy that this stem cell–rich zone is an area, which is often more severely affected in inflammatory disorders such as mucous membrane pemphigoid.

Conclusions

This is the first study to comprehensively assess the whole human conjunctival tissue, and to localize stem cells by both clonogenic ability and stem cell marker expression. The data here presented is in keeping with previous reports[10,22,34,35,40] but elucidates a much clearer depiction, whereby conjunctival stem cells are scattered throughout the tissue in the basal layer of the epithelium, but with significantly highest levels in the medial canthal and inferior forniceal areas. Furthermore, clonogenic ability and stem cell marker expression were inversely proportional to both donor age and postmortem retrieval time. The medial canthal and inferior forniceal areas may provide greater physical protection, but perhaps more importantly, are especially rich in goblet cells, intraepithelial mucous crypts, vasculature, melanocytes, and immune cells, features that are common to other stem cell niches and may constitute features of the human conjunctival stem cell niche. Additional studies are required to characterize the conjunctival stem cell niche and the mechanism of conjunctival epithelial renewal. Conjunctival biopsies should be taken from the medial canthal or inferior forniceal areas, from younger donors, and with short postmortem retrieval times to offer the greatest potential to developing stem cell–rich ex vivo expanded epithelial constructs for transplantation.

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