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Serial explant culture provides novel insights into the potential location and phenotype of corneal endothelial progenitor cells

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Abstract

The routine cultivation of human corneal endothelial cells, with the view to treating patients with endothelial dysfunction, remains a challenging task. While progress in this field has been buoyed by the proposed existence of progenitor cells for the corneal endothelium at the corneal limbus, strategies for exploiting this concept remain unclear. In the course of evaluating methods for growing corneal endothelial cells, we have noted a case where remarkable growth was achieved using a serial explant culture technique. Over the course of 7 months, a single explant of corneal endothelium, acquired from cadaveric human tissue, was sequentially seeded into 7 culture plates and on each occasion produced a confluent cell monolayer. Sample cultures were confirmed as endothelial in origin by positive staining for glypican-4. On each occasion, small cells, closest to the tissue explant, developed into a highly compact layer with an almost homogenous structure. This layer was resistant to removal with trypsin and produced continuous cell outgrowth during multiple culture periods. The small cells gave rise to larger cells with phase-bright cell boundaries and prominent immunostaining for both nestin and telomerase. Nestin and telomerase were also strongly expressed in small cells immediately adjacent to the wound site, following transfer of the explant to another culture plate. These findings are consistent with the theory that progenitor cells for the corneal endothelium reside within the limbus and provide new insights into expected expression patterns for nestin and telomerase within the differentiation pathway.

Keywords

Cornea, endothelium, limbus, progenitor, explant, nestin, telomerase, glypican-4.

The corneal endothelium resides as a monolayer of cells on the posterior surface of the cornea and maintains corneal clarity by transporting fluid from the inner corneal layers to the aqueous humor. Endothelial dysfunction therefore results in a swollen and hazy cornea, with associated vision loss. Currently, the only treatment for this condition is a full or partial thickness corneal transplant using tissue obtained from deceased donors. To overcome some of the drawbacks associated with the transplantation of donor corneal tissue, we and other groups are developing methods for growing sheets of corneal endothelium in the laboratory that could be potentially implanted into patients with endothelial dysfunction (Ozcelik *et al.*, 2013; Proulx and Brunette, 2012; Madden *et al.*, 2011). Several groups have now demonstrated the effectiveness of transplanted laboratory-produced endothelial sheets for clearing corneas in animal models of endothelial dysfunction (Mimura *et al.*, 2013), however, sufficient expansion of primary human corneal endothelial cells (HCECs) to produce these sheets is technically challenging. HCECs are considered to be non-replicative in the adult but can be induced to divide to a limited extent *in vitro* (Joyce, 2012). Unfortunately, primary cultured HCECs tend to become senescent or transition to a mesenchymal phenotype within days or after several passages depending on the donor age and culture conditions (Peh *et al.*, 2011).

Three major culture methods have been described for isolating and expanding HCECs. Konomi *et al.* (2005) favour a method in which the cells are placed straight into adherent culture following enzymatic disaggregation. Yokoo *et al.* (2005) also subject the cells to enzymatic disaggregation but then place them into a medium that promotes the formation of floating spheres. Cell monolayers are then derived from these spheres under different culture conditions. This method is called a sphere-forming assay and is proposed to promote the isolation of immature endothelial cells. The third method involves placing pieces of intact endothelium (tissue explants) into medium that promotes migration and expansion of the cells onto the plate (Mimura *et al.*, 2004). Normally, once a sufficient number of cells have grown, the cells are passaged and the original plates and explants are discarded.

We initially designed a study to compare the effectiveness of these 3 different methods for isolating and expanding primary HCECs. A cornea from a 29 year-old deceased donor was offered to us by the Queensland Eye Bank in Brisbane (with consent for research and ethical approval) for use in this study since a full thickness penetrating injury (caused by a shard of glass) had rendered the tissue unsuitable for transplant. The injury occurred 5 days prior to

death and the tissue had been stored in Optisol for 9 days prior to the study. The glass was removed and the endothelium (attached to Descemet's membrane) was carefully peeled away from the stroma and placed into stabilising medium (DMEM/F12 supplemented with 20 mM Glutamax, 20 ng/ml epidermal growth factor, 1X B27, 200 µg/ml calcium chloride and antibiotics) and incubated overnight under standard conditions (37°C, 5% CO_2). The next day a small piece of peripheral endothelium that extended into the transition zone, but excluded trabecular meshwork, was excised from the edge of the tissue and kept aside for explant culture. The rest of the tissue then underwent enzymatic disaggregation using collagenase followed by culture in medium that either promoted the formation of adherent monolayers or floating spheres. It soon became apparent that, in this case at least, the adherent and sphere culture methods were not as successful as the explant method as they failed to generate viable cultures. Therefore, at this point, we narrowed the focus of our study to the explant culture. The subsequent strategy for serial culture is illustrated in Fig 1A.

The tissue explant that was obtained from the 29 year-old donor cornea measured approximately 3 mm^2. Upon collection it immediately rolled up into a scroll with the cells on the outer surface. This typically occurs during explant preparation in our experience. The explant was incubated in a 35 mm tissue culture plate coated with fibronectin-collagen (FNC Coating Mix, Athena Enzyme Systems) using a growth medium based on that of Konomi *et al.* (2005; Optimem supplemented with 8% foetal bovine serum, 5 ng/ml epidermal growth factor, 20 ng/ml nerve growth factor, 50 µg/ml bovine pituitary extract, 0.3 mM L –ascorbic acid 2-phosphate, 200 µg/ml calcium chloride, 0.04% chondroitin sulphate, 0.1% antibiotics). It was cultured under standard conditions (37°C, 5% CO_2) with medium being refreshed twice per week. Cells were observed migrating from the explant onto the plate after several days in culture (Fig. 1B), and by day 25 a monolayer of small, compact cells had formed on the plate immediately adjacent to the explant (Fig 1C). Since the explant had yielded a large number of cells of high quality, we decided to test whether it could be used to produce another culture when transferred to a new culture plate. Therefore, after 32 days the explant was transferred to a new FNC-coated plate in growth medium for further culture. Another monolayer of small, compact cells formed around the explant in this second plate. This led us to investigate whether we may be able to utilise the explant as a continuing source of HCECs. We therefore conducted serial cultures of the explant with a culture period of 4-5 weeks between each transfer and found that it continued to produce populations of HCECs in each new plate (eg. Fig 1D). The study was terminated when the explant reached its 7th plate.

Representative photographs depicting the varying morphology of the cells that were derived from the explant are shown in Figure 1. The cells photographed were in plate 3 and were typical of all other cultures derived from the explant. In each plate, cells with a primitive-looking morphology formed a monolayer immediately surrounding the explant. These cells were small (approximately 25 µm in diameter) and had a high nucleus to cytoplasm ratio (Fig. 1E). A second population of larger cells emerged from the outer edges of the primitive cell population (Fig. 1F). The larger cells had a polygonal shape and developed as a monolayer in a cobblestone pattern, characteristic of differentiated corneal endothelial cells (Fig. 1G). When the plates were confluent, the cells were treated with trypsin for 4 to 6 minutes to loosen them from the plate for passaging. We noticed that the smaller, primitive-looking cells were extremely resistant to trypsin and remained attached to the plate after the larger cells had been removed. Fresh culture medium was therefore added to the remaining cells and, after further incubation, new sheets of more differentiated-looking HCECs formed. The populations of more primitive-looking cells therefore functioned as a reservoir for larger proliferating HCECs that could be routinely harvested in batches once confluence had been reached.

HCEC cultures that are derived from tissue explants can potentially contain stromal keratocytes. It was therefore important to investigate the cultured cells for molecular markers that would indicate their identity. Glypican-4 (GPC4) is a heparan sulfate proteoglycan that has recently been identified as a biomarker for corneal endothelial cells that is not expressed in corneal stromal cells (Cheong *et al.*, 2013). We examined GPC4 immunoreactivity in a sample of human corneal endothelium to ensure its suitability as a marker for both peripherally and centrally-located HCECs, as both of these cell populations were originally present on our cultured explant. Standard immunocytochemistry procedures were employed. Strong GPC4 immunoreactivity associated with the nuclei of endothelial cells was observed across the whole endothelial sheet, including at the extreme periphery where immature endothelial cells expressing stem cell markers have been observed previously (He *et al.*, 2012). These results confirmed the applicability of GPC4 as a general HCEC marker (Fig. 1H, I).

GPC4 was immunodetected in both primitive and differentiated cell populations established by the explant (Fig. 1J) indicating that the cells were of endothelial, rather than stromal,

origin. Interestingly, GPC4 was predominantly located in the cytoplasm of these cells, in contrast to the predominantly nuclear location observed in uncultured endothelium. The significance of the variable location for GPC4 in endothelial cells is unclear, however, it may be related to cell maturation, as passaged HCECs in confluent sheets exhibit strong nuclear expression, and very little cytoplasmic expression, of GPC4 (data not shown).

The explant-derived cells were then investigated for expression of two stem/progenitor cell markers, nestin and telomerase, to gain further insight into their status. Nestin is an intermediate filament protein that is expressed by progenitor cells in many different adult tissues (Wiese *et al.*, 2004), and is transiently expressed throughout the developing cornea during embryogenesis (Yang *et al.*, 2000). Nestin was immunodetected in the population of larger more differentiated-looking cells but not in the population of smaller cells located closest to the explant (Fig. 1J).Telomerase acts within the nucleus to maintain chromosome lengths during DNA replication and is expressed in a wide range of progenitor cells (Harrington, 2004). Telomerase can also be found in the cell cytoplasm where it may have additional activities not related to mitosis (Wojtyla *et al.*, 2011). It's activity is repressed in cells that have exited the cell cycle (Holt *et al.*, 1996). Telomerase was predominantly detected outside the nucleus of the primitive and differentiated cells that had formed a stable monolayer surrounding the explant (Fig. 1K). Relatively higher levels of telomerase immunofluorescence were observed in the nuclei of more distant cells that appeared less organised and that were presumed to be migrating and dividing (Fig. 1L). These results suggest that most cells within the monolayer immediately surrounding the explant had exited the cell cycle while those further away were still mitotic. Overall, these immunofluorescence results indicated that the explant-derived endothelial cells shared some characteristics with progenitor cells.

We were surprised to discover that nestin was not expressed in the small cells that first emerged from the explant, as these cells appeared to be more primitive, and were therefore judged to be better candidates for stem or progenitor cells, than the larger polygonal cells that they gave rise to. We hypothesised that the primitive cells in our cultures may have settled into a state of quiescence in which stem and/or progenitor marker expression was suppressed. We therefore conducted a study to analyse the expression of nestin and telomerase in a population of primitive cells that had been activated by a wound stimulus. Coincidentally, primitive HCEC were subjected to a wounding event whenever the explant was removed and

transferred to a new plate. Removal of the explant typically caused a gap in the primitive cell sheet that was filled in with new small primitive cells within 3 days (Fig. 2A, B). After 10 days there was no morphological difference between cells within and outside of the wound site (Fig. 2C). We therefore took advantage of this result to investigate expression of nestin and telomerase in primitive cells surrounding the gap initially left after explant removal. The explant was removed and the plate of cells fixed after 20 minutes. As expected, nestin was not immunodetected in the majority of small primitive cells, however, strong immunofluorescence for both nestin and telomerase was observed in the nuclei of primitive cells immediately adjacent to the wound site (Fig. 2D-F). As nestin is normally located within the cytoplasm, its presence within the nuclei of cells at the wound site was intriguing. Nestin has also been observed in the nuclei of mitotic human tumour cells (Veselska *et al.*, 2006) and basal pterygium epithelial cells (Tonthat and Di Girolamo, 2013), the latter of which are suggested to be progenitors undergoing mitosis. Taken together, the observation of both telomerase and nestin in the nuclei of primitive cells at the wound edge indicated that these cells were were capable of responding to a wound stimulus by rapidly upregulating stem/progenitor markers, undergoing mitosis, and regenerating the cell sheet.

It has been suggested that cells with stem or progenitor characteristics may reside in the posterior limbus of the human cornea. McGowan *et al.* (2007) have identified nestin and telomerase positive cells within the trabecular meshwork and transition zone of the adult cornea limbal region. They hypothesise that these may be the source of slow moving streams of cells that have the potential to replace lost or damaged cells in the central cornea. This hypothesis is supported by morphological evidence showing centripetally-arranged streams of cells extending from the extreme periphery towards the central cornea (He *et al.*, 2012). We suggest that the nestin-positive corneal endothelial cells that differentiated from the pools of more primitive cells in our plates are equivalent to those nestin-positive cells observed in the limbus *in vivo*. We further suggest that the more primitive cells that upregulated nestin and telomerase in response to wounding in our cultures represent a more immature population of corneal endothelial stem-like cells that have yet to be observed directly *in vivo*. Intriguingly McGowan *et al.* (2007) noticed that a larger set of stem cell markers could be detected in the limbal region of wounded corneas compared with the set detected in unwounded corneas. Specifically, Oct3/4 and Wnt-1, as well as nestin and telomerase, were detected in wounded corneas. Based on this data, they proposed that there may be limbally-located endothelial stem cells within the cornea that become activated in response to

wounding. The design of their study did not allow them to determine whether injury was likely to stimulate a pre-existing population of nestin-expressing cells to co-express additional stem cell markers, or whether a different population of cells were being stimulated. Based on our results we suggest that progenitors at different stages of maturation are present within the limbus of the human cornea. Very immature progenitors do not normally express nestin but can be stimulated to express it and other stem cell markers and to become proliferative in response to wounding. More mature progenitors do normally express nestin and these may be the source of HCECs that slowly migrate towards the central cornea throughout adulthood.

In conclusion, this case study describes a novel method for isolating and expanding HCECs with progenitor cell characteristics from corneal endothelium through serial explant culture. Our results support and expand upon previous studies that indicate that stem or progenitor endothelial cells, that are capable of responding to a wound stimulus, reside in the adult corneal limbus. In addition, our data suggest that progenitors at different maturation stages, with distinct morphological and molecular characteristics, may be present in the adult limbus. Due to their proliferative potential, endothelial stem or progenitor cells have enormous potential therapeutic value for patients with vision loss caused by low endothelial cell density. It is therefore important to know where these cells are in the adult cornea, what their normal roles are and whether they can be stimulated to regenerate the endothelium. The present study provides a basis from which to further investigate these questions.

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Figure Legends.

Figure 1. Demonstration of serial cultures derived from an explant of corneal endothelium. (A) Schema illustrating the method used to generate multiple cultures of primary non-passaged human corneal endothelial cells from an individual endothelium explant taken from the cornea of a 29 year old donor. The explant was transferred to 7 new plates over a period of 6 months, generating sheets of small, primitive cells in each plate. (B) The endothelium explant in its first plate, after 4 days in culture. Cells are seen migrating out from one end of the explant. (C) A densely-packed sheet of small cells surrounds the explant at 25 days. (D) This figure shows the explant in its 4th plate, 154 days after isolation from the cornea. (E) A sheet of small, primitive cells generated by the explant in its 3rd plate. Under close examination the cells exhibit a high nucleus to cytoplasm ratio (see 3x enlarged inset). (F) Primitive cells are arranged in a colony-type formation that surrounds the explant. A population of larger cells emerge from the margins of this colony. The transition between small (on the left) and larger cells (on the right) is evident in this figure. (G) Larger cells generated at the edges of the primitive cell population are polygonal and form a monolayer in a cobblestone pattern typical of corneal endothelial cells. (H) A sample of fixed human endothelium taken from a similar corneal location to that of the serially-cultured explant. The tissue has been photographed under phase contrast and Hoechst-stained nuclei appear blue. Cells towards the centre of the endothelium (to the left of photo) are densely packed while the cells towards the extreme periphery (to the right of photo) are less numerous and irregularly-spaced. (I). Corresponding fluorescence image to that in 1K. Cell border expression of ZO-1 is patchy at the extreme periphery of the corneal endothelium (to the right of photo). GPC4 immunoreactivity is detected in central and peripheral endothelial cells. (J) Immunodetection of GPC4 in cells generated by the explant indicates that they are of corneal endothelial origin. The stem/progenitor cell marker nestin is immunodetected in the larger cell population. (K) Telomerase is predominantly immunodetected in the cytoplasm of both the small and large cells immediately surrounding the explant. (L) Telomerase is immunodetected in the nuclei of migratory cells at the edges of the expanding cell population. Primary antibodies: GPC4, Aviva Systems Biology ARP64505; ZO-1, Invitrogen 339100; telomerase, Novus Biologicals NB110-89471SS; nestin, R&D Systems MAB1259. Scale bars = 50µm.

Figure 2. Upregulation of stem cell markers following wounding of the explant culture. (A) Removal of the explant results in a wound in the cell sheet. (B) The cell sheet is repaired by 3 days following the injury, however, some cells within the wound site appear morphologically-different to surrounding cells (arrow). (C) At 10 days following the injury a morphologically-uniform cell layer covers the former wound site. (D-F) A cell culture was fixed 20 minutes after removing the explant and stem/progenitor marker expression investigated by double immunofluorescence. (D) The edge of the wound site is indicated by white dotted lines and cell nuclei are indicated by Hoechst staining. White circles indicate nuclei that are immunoreactive for nestin (E) and telomerase (F). Scale bars = 50µm.

Figure(s)

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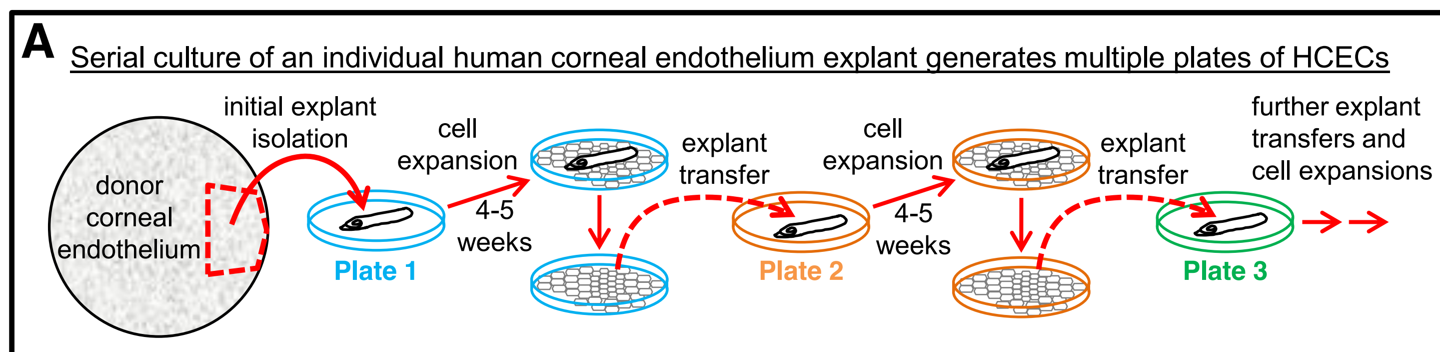
Figure 1



B Day 4, plate 1

C Day 25, plate 1

D Day 154, plate 4

E

F

G

H

I GPC4 ZO-1

J GPC4 nestin

K telomerase

L telomerase

Figure 2