Elucidation of the migratory behaviour of the corneal endothelium

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CHAPTER 5

Asymmetrical endothelial cell migration from *in vitro* Quarter-Descemet membrane endothelial keratoplasty grafts

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ABSTRACT

Purpose: To investigate *in vitro* central and peripheral corneal endothelial cell (EC) migration from Quarter–Descemet membrane endothelial keratoplasty (Quarter-DMEK) grafts.

Methods: Quarter-DMEK grafts were obtained from 10 corneas ineligible for transplantation but with intact and viable ECs. Ten Quarter-DMEK grafts were 'sandwiched' between two glass slides and cultured over 1 week in a humidified atmosphere at 37°C and 5% CO_2. Cell migration was evaluated by light microscopy at standardized time intervals. In addition, immunohistochemistry analyses were performed to assess the detailed structural organization of ECs in the corneal centre and far periphery.

Results: Endothelial cell (EC) migration occurred from the radial cut graft edges, but not from the far peripheral area. Cell migration followed three different migration patterns: (1) individual cell migration, (2) uncoordinated cell migration of cell clusters and (3) collective migration in which ECs moved as a sheet. Immunostaining showed the presence of ECs up to the far periphery but with different expression patterns of phenotypical markers ZO-1, Na^+/K^+ –ATPase and vimentin compared to central ECs.

Conclusion: *In vitro* EC migration from Quarter-DMEK grafts occurs along the radial cut edges with a decrease in migration activity towards the corneal far periphery. No migration occurred along the outer peripheral corneal edge possibly due to a different anatomical matrix in the far periphery. Hence, ECs from the far periphery may not contribute to corneal clearance of the adjacent bare area after Quarter-DMEK surgery, but these cells may constitute a valuable cellular reserve on the graft.

Introduction

Recently, we have introduced several modifications of Descemet membrane endothelial keratoplasty (DMEK) including Quarter-DMEK, a technique that potentially allows to retrieve four quadrants from a full-size Descemet membrane (DM) and therefore to utilize four endothelial grafts from a single donor cornea.[1–4] In a first series, Quarter- DMEK eyes showed visual outcomes similar to conventional (circular) DMEK.[4] At the slit lamp, however, Quarter-DMEKeyes typically showed a different corneal clearance pattern with clearing primarily occurring adjacent to the radial cut graft edges but not along the 'limbal' round edge of the Quarter-DMEK grafts. This finding would suggest that donor endothelial cell (EC) migration varies over these grafts, with an almost complete absence of migration in the farperipheral anatomical area of DM.

The aim of this study was to further evaluate how EC migration may vary over different anatomical corneal areas,by studying *in vitro* EC migration fromorgan-cultured Quarter-DMEK grafts,and to determine how Quarter-DMEK grafts may be positioned best onto the posterior recipient corneal surface during surgery in order to obtain a homogenous redistribution of donor ECs postoperatively.

Materials and Methods

Corneas

Ten human corneas ineligible for transplantation but with an intact and viable EC layer were obtained from seven donors (mean age 72 (±13) years; range 51–84 years; **Table 1**). All donors had stated to have no objection against transplanted-related research.

Quarter–Descemet membrane endothelial keratoplasty graft preparation

Quarter-DMEK grafts for these experiments were prepared at Amnitrans EyeBank Rotterdam as previously described.[4] Briefly, after decontamination of the globes, corneo-scleral rims were excised within 36 hours post-mortem. After EC morphology and viability were evaluated and digital photographs were made with inverted light microscopy (Zeiss Axiovert 40C; Carl Zeiss International, Zaventem, Belgium), the excised corneo-scleral rims were stored in organ culture medium (CorneaMax; Eurobio, Courtaboeuf, France) at 30°C until further processing. To peel the Quarter-DMEK grafts, corneo-scleral rims were placed endothelial-side-up on a custom made holder with a suction cup. The endothelium was then stained for visualization with 0.04% hypotonic trypan blue solution (Hippocratech, Rotterdam, the Netherlands) for 10 second. Next, the DM-EC sheet including trabecular meshwork (TM) was loosened over 360° from the scleral spur towards the corneal centre and the corneo-scleral button was divided into four equally sized parts with a surgical blade (no. 24 knife; Swann-Morton, Sheffield, UK). The DM was then centripetally stripped from the posterior stroma by grasping the TM with McPherson forceps (Moria, Medical Workshop, Groningen, the Netherlands), thereby obtaining four Quarter-DMEK grafts. After stripping, four rolls formed spontaneously with the endothelium on the outer side and the TM still attached to facilitate later graft handling. Endothelial cell morphology and viability were again assessed, and images of each Quarter-DMEK graft were evaluated using the fixed frame method. For all ten corneas, the endothelial cell density (ECD) determined in the eye bank after DMEK graft preparation was on average 2743 (±185) cells/mm^2, with no significant ECD difference between the four quarters ($P > .05$) deriving from the same cornea. Each Quarter-DMEK graft was then stored separately in organ culture medium (CorneaMax; Eurobio) before being evaluated for chemotactic cell ability or immunohistochemistry analysis.

Table 1. Donor demographics.

Donor Information	Indicators
Donor data	
Gender	
Female	4
Male	3
Mean age (±SD), yrs. (range)	72 (±13), (51–84)
Mean storage time (±SD), days (range)	11 (±5), (3–16)
Cause of death	
Cardio/Stroke	2
Respiratory	5

*Mean storage time = time between death and culture of first isolated DM-EC tissue; SD = standard deviation; yrs. = years

Cell migration study

To analyse cell migration patterns of the corneal endothelium on a Quarter-DMEK graft, 10 individual Quarter-DMEK rolls with the TM attached, obtained from 10 different corneas, were unfolded endothelial-side-up on a FNC-coated (fibronectin, collagen and albumin coating mix; Athena ESTM Baltimore, MD, USA) glass coverslip and evaluated *in vitro*. Unfolding for all grafts was performed in a 'no-touch' manner by grabbing the graft only at the TM site with a McPherson forceps and dropping organ culture medium onto the graft while the glass coverslip was kept tilted under a small angle. Next, the TM was carefully removed from the Quarter-DMEK grafts, and the endothelium was submerged in serum containing culture medium to ensure cell viability during the experiments. Serum containing culture medium consisted of 15% fetal bovine serum in Dulbecco's modified Eagle's medium supplemented with 2 mM L-Glutamine, 2 ng/ml fibroblast growth factor (bFGF), 0.3 mM L-ascorbic acid 2-phosphate (all from Sigma-Aldrich, Zwijndrecht, the Netherlands) and 10 000 U-ml Pen/ Strep (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). A second FNC-coated glass coverslip that was spatially separated from the flattened Quarter-DMEK graft by a suture wire (Supramid TS194-0, non-absorbable, Hueber Medica) was then carefully placed on top. The Quarter-DMEK graft, now 'sandwiched' between the two glass slides, was then transferred to a 24-well plate, and kept over 9 days in a humidified atmosphere at 37°C and 5% CO_2. For routine maintenance, medium was replaced with fresh culture medium every 2–3 days. To assess cell morphology and the degree of cell migration, the Quarter-DMEK grafts were photographed daily with an AxioVert.A1 microscope with AxioCam 305 color camera (Zeiss, Oberkochen, Germany).

Immunohistochemistry

To evaluate the expression of continuous zonula occludens-1 (ZO-1) at the cell–cell borders, thin cortical vimentin cytoskeleton and pump function through Na^+/K^+ –ATPase, immunohistochemistry analysis was performed at room temperature on Quarter-DMEK grafts obtained from the same corneas as the grafts used for the cell migration experiments. Quarter-DMEK grafts were unfolded and flattened on silane-precoated glass slides (Sigma Aldrich) before fixation in 4% paraformaldehyde (Sigma-Aldrich) for 30 min. Following fixation, the

grafts were first washed with phosphate buffered saline (PBS), then permeabilized using permeabilization buffer (0.1% Triton X-100 in PBS; Sigma Aldrich) and finally incubated with blocking buffer (5% bovine serum albumin in PBS; Sigma-Aldrich) for 1 hour to prevent non-specific staining. Blocking buffer was also used for primary and secondary antibody (Life Technology, Bleiswijk, the Netherlands) dilutions. Incubation with primary antibodies anti-ZO-1 tight junction protein (anti-ZO-1/TJP1; dilution 1:100), anti-vimentin filamentous protein (anti-vimentin, dilution 1:100) and anti-sodium/potassium-ATPase (anti-Na^+/K^+ –ATPase, dilution 1:100) was performed for 1 hour and was followed by several PBS washing steps. Samples were then incubated with secondary antibodies (dilution 1:200) for 1 hr. As control, an antibody to smooth muscle actin (anti-α-SMA, dilution 1:100) was included as a marker for smooth muscle cells and myofibroblasts. After washing with PBS, the samples were stained with 40,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) to visualize the nuclear DNA and then imaged using an inverted fluorescence microscope connected to a camera (Axiovert; Zeiss).

Results

Structural analysis of endothelial cell distribution on Quarter-DMEK grafts

With light microscopy, all Quarter-DMEK grafts showed an intact endothelium up to the radial cut edges (**Figure 1A**).

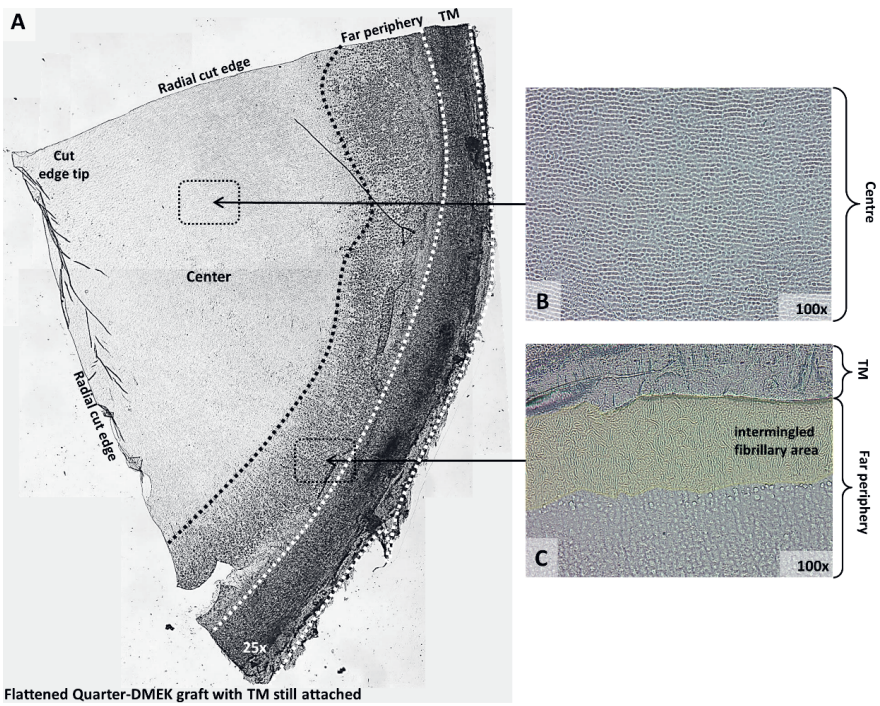

Figure 1| General view of a Quarter-Descemet membrane endothelial keratoplasty (DMEK) graft flattened on a glass support. (A) Overview of a flattened Quarter-DMEK graft with the trabecular meshwork (TM) still attached. Corneal centre (B) and far periphery (C) show structural differences, which become more distinct when displayed at higher magnifications (x100). (B) Corneal centre with closely packed hexagonal endothelial cells. (C) Far peripheral area is dominated by a fibrillary area adjacent to the TM.

From the central to far peripheral corneal areas, the ECs showed a different morphology, with a relatively homogenous cell distribution in the corneal centre and less densely packed cells with a more heterogeneous morphology towards the periphery (**Figure 1A–C**). In the area directly adjacent to the TM, collagen fibres intermingled with the ECs in a spiral-like pattern (**Figure 1C**).

Cell migration study

All grafts showed substantial cell migration from day 4 up to day 6 with EC migration along the radial cut edges up to the central tip of the graft **(Figures 2** and **3**). The degree of migration decreased towards the peripheral graft area. Over the far periphery, that is the intermingled fibrillary area, no cell migration was observed, that is no cells crossed the rounded graft edge at any time-point (**Figures 2B** and **3A–C**).

At the radial cut edges, three types of cell migration patterns could be observed: (1) individual cell migration in an exploratory manner lacking a directional stimulation (i.e. random cell migration) was present in five of 10 grafts (**Figure 2C**), (2) migration of cell clusters, with cells coexisting at the leading migratory edge, not forming a continuous monolayer (i.e. uncoordinated cell migration) was observed in four of 10 grafts (**Figure 2D–G**) and (3) migration of interconnected cells that collectively departed the DM with a leading 'cell group' at the front edge (i.e. collective cell migration) was observed in one of 10 graft (**Figure 3D–I**).

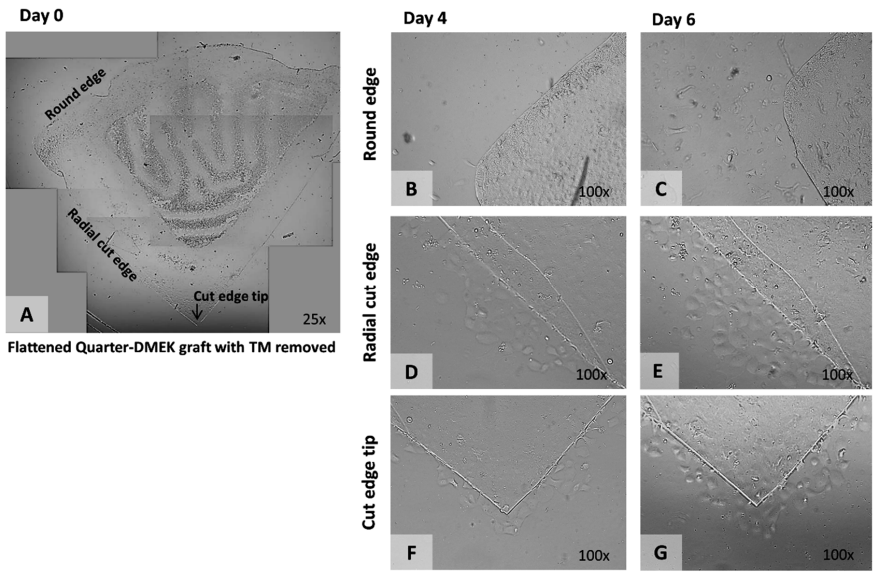

Figure 2| Example of individual and uncoordinated *in vitro* endothelial cell (EC) migration. (A) Collage of light microscopy images (x25 magnification) to create an overview of a flattened Quarter-Descemet membrane endothelial keratoplasty (DMEK) graft without trabecular meshwork attached, at the start of the cell migration experiment (Day 0). (B–G) Light microscopy images of the round edge (B,C), the radial cut edge (D,E) and the cut edge tip (F,G) of the Quarter-DMEK graft taken at Day 4 (left) and Day 6 (right) with x100 magnification. (B) In the area of the round edge of the Quarter-DMEK graft, no apparent cell migration is observed across the round graft edge with (C) only individual cells migrating across the far peripheral cut edge of the graft. (D,E) Along the radial cut edge of the Quarter-DMEK graft, single bleb-like ECs migrating onto the glass coverslip are observed. (F,G) At the cut edge tip of the Quarter-DMEK graft, individual cells migrate onto the glass coverslip.

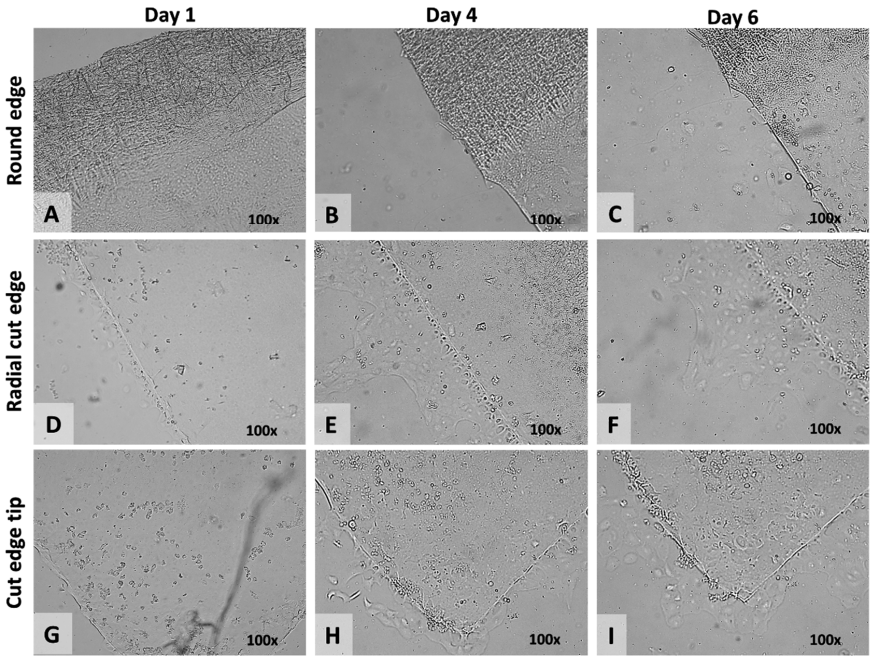

Figure 3| Example of collective *in vitro* endothelial cell (EC) migration. (A–I) Light microscopy images of the round edge (A–C), the radial cut edge (D–F) and the cut edge tip (G–I) of the Quarter-Descemet membrane endothelial keratoplasty (DMEK) graft taken at Day 1 (left), Day 4 (middle) and Day 6 (right) with x100 magnification. (A–C) In the area of the round edge and far periphery of the Quarter-DMEK graft, no EC migration onto the glass slide was observed up to Day 6. (D–F) Along the radial cut edges of the Quarter-DMEK graft, collective EC migration in a form of a monolayer was observed; leader cells at the front edge of the advancing cell sheet are identifiable. (G–I) Around the cut edge tip of the Quarter-DMEK graft, the collective migration pattern was most evident at Day 6.

Immunohistochemistry

Immunohistochemistry analysis confirmed the presence of ECs up to the far peripheral area of the Quarter-DMEK grafts, that is up to the round edge of the graft (**Figure 4**). However, ECs in the centre and far periphery revealed different expression patterns of the typical endothelial markers ZO-1, vimentin, and Na^+/K^+ –ATPase (**Figure 4**). While ZO-1 expression at the apical junctions in the central graft area showed the typical hexagonal cell borders, the distribution of ZO-1 towards the intermingled fibrillary area in the far periphery was more discontinuous and revealed larger cells than in the central area (**Figure 4A,B**). Differences in cell size and shape between ECs in the centre and far periphery were also shown by the expression of vimentin that showed a mat of filaments within the EC cytoplasm in the far periphery (**Figure 4C,D**). Towards the far periphery, also Na^+/K^+ –ATPase pumps were expressed more irregularly (**Figure 4E,F**). The absence of α-SMA-positive cells in both central and peripheral regions of the Quarter-DMEK graft verified the absence of any transformed ECs (**Figure 4G,H**).

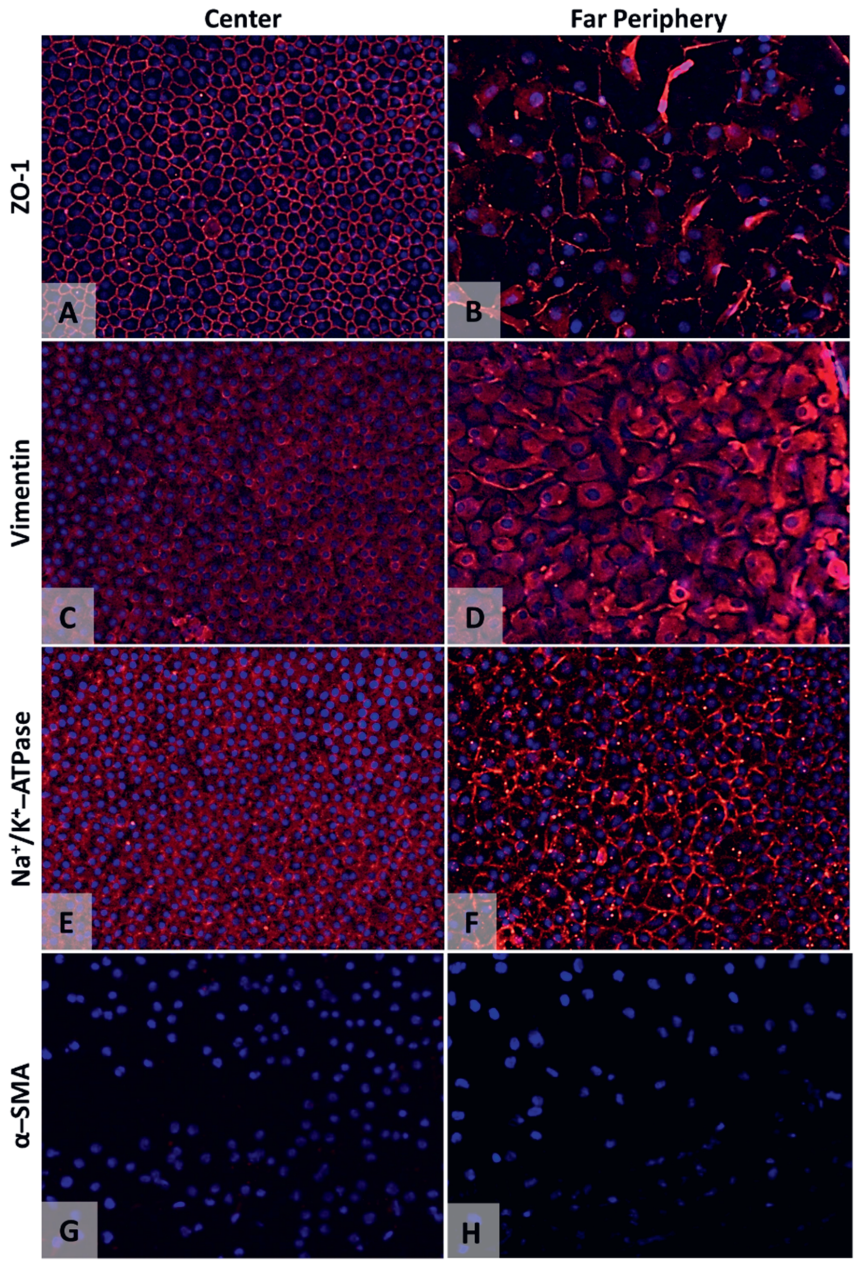

Figure 4| Immunofluorescence staining of the Quarter-Descemet membrane endothelial keratoplasty (DMEK) graft in the centre compared to the far periphery. Expression of ZO-1 (A,B), vimentin (C,D), Na^+/K^+ –ATPase (E,F) and α-smooth muscle actin (α-SMA; G,H) was analysed. The central endothelium showed characteristic expressions for the tight junction protein ZO-1 (A, red), structural protein vimentin (C, red) and functional protein Na^+/K^+ –ATPase (E, red) counter- stained with DAPI (blue). The presence of markers in the far peripheral area (B,D,F) verified the presence of endothelial cells (ECs) up to the round edge of the Quarter-DMEK graft. However, the cells in the far periphery showed a different expression pattern for these endothelial markers (B,D,F red) as compared to the central area. α-SMA, used as a negative control for the ECs, was absent in the centre and in the far periphery of the endothelium (G,H). x200 magnification.

Discussion

In this study, we evaluated how EC migration may vary over different anatomical corneal areas, by studying *in vitro* EC migration from organ cultured Quarter-DMEK grafts.

Our results showed that corneal ECs migrate from the radial cut edges but not from the round edge of a Quarter-DMEK graft, that is the far, 'limbal' periphery of DM. The lack of EC migration from the peripheral round edge may be explained by the structural organization of the peripheral DM. Immunolocalization showed expression of the structural (ZO-1 and vimentin) and functional (Na^+/K^+–ATPase) markers up to the far periphery, however, although ECs in the far periphery formed a cellular monolayer, these cells did not show the typical hexagonal cell structure.

He et al. (2012) showed that in the corneal far periphery, ECs were organized in small radial rows induced by the furrow-like distribution of the underlying collagen fibres. It was suggested that this anatomical organization is to direct the migration of ECs from specific niches in the far periphery towards the centre of the cornea throughout life, limiting the migration in the other direction. Hence, if migration would not occur or is limited from the limbal edge of a Quarter-DMEK graft, it may be important to position the graft eccentrically, with its radial cut edges near the pupillary area while the peripheral round edge is positioned peripherally, to avoid slowly resolving corneal oedema in the visual axis.[5] Further, it may be beneficial for the smaller Quarter-DMEK graft – compared to a circular conventional graft – that not all cells are able to 'leave' the graft. This could possibly enable early stabilization of the EC density over time.

Although detailed knowledge about the movement of corneal ECs is lacking, migration of other cell sheets has been studied more extensively, especially *in vitro*.[6–10] Under normal conditions, cells maintain strong adhesions with neighboring cells. When a wound is created, the released transient chemical signals enhance cellular motility near the edge of the wound and cells at the wound edge extend large polarized lamellipodia towards the free surface producing an overall traction force that is directed towards the wound.[8–10] Another important denominator of cell migration is growth factor signaling, which is necessary for directional migration.[6] For instance, *in vitro*, fibroblast growth factor 2 (FGF-2, bFGF), has been shown to induce human umbilical vein ECs near the boundary of a sheet to move into open space, whereas in the absence of bFGF, cells migrated with normal speed but failed to sense open space or to respond with directed movement.[6]

While collective migration patterns were observed for other cell types, in our study, uncoordinated or individual migration patterns were more prevalent, which might be partly explained by the experimental set-up that might induce a limited number of viable neighboring cells, for example due to cell damage during tissue handling. However, differences in cell migration from the radial cut edges and from the peripheral round edge were clearly distinguishable.

Recent studies on the effect of Rho associated kinase (ROCK) inhibitors showed that topical administration of ROCK-inhibitors after induced surgical injury of rabbit corneal endothelium triggered cell adhesive changes which contributed to enhanced proliferation and migration.[11] A similar observation was made after cases of surgical 'Descemetorhexis only' procedures, that is stripping of a diseased DM–endothelium layer without subsequent corneal graft transplantation, followed by topical administration of ROCK-inhibitors (Moloney et al. 2017). Although, corneas after Quarter-DMEK may show sufficient clearance (Zygoura et al. 2018), ROCK-inhibitors may potentially enhance EC migration and corneal clearance. However, as ROCK-inhibitors were not administered after *in vivo* Quarter-DMEK surgery, they were also not added as an agent to the serum-containing growth media in our experiments to ensure better comparability of *in vitro* and *in vivo* EC migration patterns.

In conclusion, asymmetrical EC migration of Quarter-DMEK grafts may explain the corneal clearance pattern after Quarter-DMEK surgery, with cell migration predominantly from the radial cut edges, but not the rounded, limbal edge. While the ECs from the graft's far periphery may not contribute to corneal clearance after Quarter-DMEK surgery, these cells may constitute a valuable cellular reserve on the graft.

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