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Transplantation of cultured corneal endothelial cells: Towards clinical application

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CHAPTER 5

Adapted from:

Evaluation of the Suitability of Biocompatible Carriers as Artificial Transplants Using Cultured Porcine Corneal Endothelial Cells

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ABSTRACT

Purpose/Aim: Evaluating the suitability of bioengineered collagen sheets and human anterior lens capsules (HALCs) as carriers for cultivated porcine corneal endothelial cells (pCECs) and *in vitro* assessment of the cell-carrier sheets as tissue-engineered grafts for Descemet membrane endothelial keratoplasty (DMEK).

Materials and Methods: pCECs were isolated, cultured up to P2 and seeded onto LinkCell™ bioengineered matrices of 20 μm (LK20) or 100 μm (LK100) thickness, and on HALC. During expansion, pCEC viability and morphology were assessed by light microscopy. ZO-1 and Na^+/K^+-ATPase expression was investigated by immunohistochemistry. Biomechanical properties of pCEC-carrier constructs were evaluated by simulating DMEK surgery *in vitro* using an artificial anterior chamber (AC) and a human donor cornea without Descemet membrane (DM).

Results: During *in vitro* expansion, cultured pCECs retained their proliferative capacity, as shown by the positive staining for proliferative marker Ki67, and a high cell viability rate (96 ± 5%). pCECs seeded on all carriers formed a monolayer of hexagonal, tightly packed cells that expressed ZO-1 and Na^+/K^+-ATPase. During *in vitro* surgery, pCEC-LK20 and pCEC-LK100 constructs were handled like Descemet stripping endothelial keratoplasty (DSEK) grafts, i.e. folded like a "taco" for insertion because of challenges related to rolling and sticking of the grafts in the injector. pCEC-HALC constructs behaved similar to the DMEK reference model during implantation and unfolding in the artificial AC, showing good adhesion to the bare stroma.

Conclusions: *In vitro* DMEK surgery showed HALC as the most suitable carrier for cultivated pCECs with good intraoperative graft handling. LK20 carrier showed good biocompatibility, but required a DSEK-adapted surgical protocol. Both carriers might be notional candidates for potential future clinical applications.

KEYWORDS: porcine endothelial cells, cell culture, donor material, endothelial cell transplantation, cell carriers.

INTRODUCTION

Corneal endothelial cells (CECs) are essential for the preservation of corneal transparency, since loss of their functionality following endothelial diseases or trauma eventually leads to corneal swelling and edema.[1] Because mature human CECs (hCEC) do not replicate *in vivo*,[1–4] replacement of diseased or damaged endothelium by corneal transplantation is currently the only treatment option.[5] To date, Descemet membrane endothelial keratoplasty (DMEK) specifically replaces the recipient's diseased or damaged endothelium (EC) and Descemet membrane (DM) with the same layers of a healthy donor.[6,7] DMEK provides faster and better visual rehabilitation than other transplantation techniques; however, its application is limited by the shortage of high-quality healthy donor tissue.

One approach to target the shortage of endothelial grafts relies on the *in vitro* expansion of CECs on suitable cell carriers. These tissue-engineered endothelial grafts could become an alternative to the availability of human donor tissue.[8] Previous *in vitro* studies have reported the use of denuded DM and human anterior lens capsules (HALCs), as well as bioengineered matrices consisting of collagen or gelatin, or a combination of biopolymers, as potential carriers for cultured CECs.[9–17] These cell-carrier constructs should preferably be similar to an "original" DMEK-graft, since it has been reported for Descemet stripping (automated) endothelial keratoplasty (DSEK/DSAEK) that thicker grafts may interfere with visual outcome[18] and the biomechanical properties of cell-carrier constructs should allow implantation.

In this study, we evaluated collagen-based bioengineered scaffolds and HALC as carriers for viable cultured porcine corneal endothelial cell (pCEC) sheets. The suitability for transplantation of the pCEC-carrier constructs with the DMEK surgical protocol was tested by simulating DMEK surgery *in vitro*.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS), Dulbecco's phosphate-buffered saline (PBS), ascorbic acid 2-phosphate (Asc-2P), paraformaldehyde (PFA), L-Glutamine, trypsin, ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA), 4′,6-diamidino-2-phenylindole (DAPI), and Triton X-100 were purchased from Sigma-Aldrich Chemistry BV (Zwijndrecht, The Netherlands). Chondroitin sulphate and Pen/Strep Pre-Mix were purchased from Carl Roth GmbH$^+$ Co. KG (Karlsruhe, Germany). Fibronectin, collagen, and albumin (FNC) coating mix was purchased from Athena ESTM (Baltimore, MD, USA). Opti-MEM™ I Reduced Serum Medium (Opti-MEM), bovine pituitary extract (BPE), anti-ZO-1/TJP1 primary antibody, secondary antibodies, and ReadyProbes™ Cell Viability Imaging Kit (Blue/Green) were obtained from Life Technology Europe BV (Bleiswijk, The Netherlands). Anti-Na^+/K^+-ATPase primary antibody and anti-Ki67 primary antibody were obtained from Abcam (Cambridge, United Kingdom). Betadine was obtained from Hippocratech (Rotterdam, The Netherlands). Hypotonic Trypan Blue solution 0.04% (Hippocratech, Rotterdam, The Netherlands) was used to stain the pCEC-carrier constructs during *in vitro* preparation and surgeries. LinkCell™ collagen bio-membranes were provided by LinkoCare Life Sciences AB (Linköping, Sweden).

Porcine corneal endothelial cell isolation

A total of 15 porcine eyes were collected from a local slaughterhouse within 24 h after death. First, porcine globes were decontaminated as described for human eyes[19] and adapted for porcine eyes, including an additional betadine-treatment step before decontamination. Next, the corneo-scleral rim was excised under sterile conditions, placed endothelial side-up in one well of a 12-well plate, and treated with 0.05% Trypsin/ 0.02% EDTA (TE) solution for 20 min at 37°C to cut away the focal adhesion from anchoring the endothelial cells to DM. Trypsin activity was stopped by addition of Opti-MEM containing 8% FBS directly on the corneo-scleral rim. Then, the enzymatically dislodged cells were collected with a sterile pipette tip and centrifuged at 500 rpm for 8 min at 37°C. Based on

the protocol described by Proulx et al.,[20] cells were then re-suspended in fresh Opti-MEM containing 8% FBS, 4 mM L-Glutamine, 200 mg/ml Ca^{2+} chloride, 50 μg/ml BPE, 0.3 mM Asc-2P, 0.08% chrondroitin sulphate, and 100 IU/ml Pen/Strep Pre-Mix (hereafter referred to as "culture medium") and plated in 3 wells of a 6-well plate previously coated with FNC coating mix. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. For routine maintenance, every 2–3 days the culture medium was replaced. When primary cultures of pCECs reached 80–90% confluence (approximately after 1 week), they were passaged by treatment with TE solution for 10 min at 37°C and 5% CO_2. Next, the cell suspension was collected, centrifuged at 500 rpm for 8 min at 37°C and the cell pellet was re-suspended in fresh culture medium. The pCECs were sub-cultured at a 1:2 splitting ratio on FNC-coated culture well plates and their morphology at confluence and during expansion was evaluated with an AxioVert.A1 microscope with AxioCam ERc 5s stand-alone functionality camera (Zeiss, Oberkochen, Germany). Use of animal tissue in this study followed the institution's guidelines for animal tissue-based research.

Cell proliferation and viability

Ki67 is a sensitive marker for cell proliferation.[21] To detect Ki67 expression, pCECs at P2 were cultured for 1 week on glass coverslips and fixed in 4% PFA for 15 min at room temperature (RT). Following fixation, samples were washed with PBS containing 0.1% Triton X-100 and then incubated with blocking buffer (3% BSA in PBS) for 30 min at 37°C to prevent non-specific staining. Blocking buffer was also used for primary and secondary antibody dilutions. Samples were incubated with primary antibody anti-Ki67 (dilution 1:100) for 1 h at 37°C and were subsequently washed several times with PBS. Next, samples were incubated with second antibodies (dilution 1:200) for 1 h at 37°C. After washing with PBS, samples were stained with DAPI to visualize the nuclear DNA, and then imaged using an inverted fluorescence micro-scope connected to a camera (Axiovert, Zeiss).

Cell viability was determined on pCECs at P2 cultured on glass coverslips by using the ReadyProbes™ Cell Viability Imaging Kit. Briefly, two drops of each stain (NucBlue® Live reagent and NucGreen® Dead reagent, respectively) were

added per ml of culture media and samples were incubated for 15 min at 37°C in a humidified atmosphere containing 5%CO_2. The cell viability rate was calculated as the average in three areas, each measuring 0.01 mm^2, on digital microphotographs. The percentage of viable cells was determined by manual counting according to the fixed-frame method.

Human anterior lens capsule isolation

HALCs were isolated from human donor eyes at Amnitrans Eyebank Rotterdam. In all cases, the donors had stated to have no objection against transplant-related research. While holding the HALC with the anterior pole up with forceps, an incision into the lens' equatorial area was made using a surgical blade to accomplish a 360° cut. The obtained HALCs were spread epithelial side-up and the lens cortex which was still adherent to the posterior lens cortex was removed using a surgical sponge (Simovision BV, Diemen, The Netherlands) soaked in 70% ethanol. Then, the HALCs were trephined to the desired size (Ø 8.0 mm) from the interior side, and treated with TE solution for 20 min. Next, HALCs were flattened and the loosely attached lens epithelium was removed by sweeping using a sponge soaked in BSS. After rinsing with BSS, because of the elastic properties of the membrane, a "HALC roll" formed spontaneously with the epithelial side on the inside. Each HALC was stored in a glass vial containing 5 ml PBS at 4°C until further use. When ready for cell seeding, the lens capsules were spread with the epithelial side down[16] over FNC-coated glass coverslips in 24-well tissue culture dishes and kept moist in PBS.

Seeding of cultured pCECs on biocompatible carriers

The selected carriers differed regarding their artificial extra cellular matrix (ECM) composition, technological process, and biophysical (strength of cell-substrate adhesion or cell proliferation enhancement) and biomechanical properties (e.g. tensile strength, elasticity). Three different carriers were evaluated in this study: LinkCell™ collagen I bio-membranes of 20 μm and 100 μm thickness (LK20 and LK100, respectively)[22] and HALCs. Diameter of the LK20 and LK100 for the experiments was 9.5 mm.

During the storage time, the carriers were kept hydrated in PBS in order to preserve their biomechanical properties. Primary pCECs, after reaching 80–90% of confluence, were passaged twice (up to P2) before they were seeded onto each the three carriers (LK20, LK100, and HALC). All pCEC-carrier constructs were cultured up to 1 week before they were used for the *in vitro* surgeries.

Immunofluorescence

ZO-1 and Na^+/K^+-ATPase are phenotypical markers for CEC.[23,24] To visualize ZO-1 and Na^+/K^+-ATPase, pCECs at P2 were cultured for 1 week either on glass coverslips or directly on the biocompatible carriers and fixed in 4% PFA for 15 min at RT. Following fixation, samples were washed with PBS containing 0.1% Triton X-100 and then incubated with blocking buffer (1% BSA in PBS) for 1 h at 37°C to prevent non-specific staining. Blocking buffer was also used for primary and secondary antibody dilutions. Incubation with primary antibodies anti-ZO-1 tight junction protein (anti-ZO-1/TJP1, dilution 1:100) and anti-sodium/potassium-ATPase (anti-Na^+/K^+-ATPase, dilution 1:100) was per-formed for 1 h at 37°C and was followed by several PBS washing steps. Next, samples were incubated with secondary antibodies (dilution 1:200) for 1 h at 37°C. After washing with PBS, the samples were stained with DAPI to visualize the nuclear DNA, and then imaged using an inverted fluorescence microscope connected to a camera (Axiovert, Zeiss).

***In vitro* surgeries**

In order to assess suitability for transplantation, surgeries with the pCEC-carrier constructs were simulated *in vitro* by using human anterior remnants (donor corneo-scleral rim without DM and endothelium; donors had stated to have no objection against transplant-related research) mounted onto an artificial anterior chamber (AC; DORC International, Zuidland, The Netherlands).[25] The ability of the pCEC-carrier constructs to form a roll in BSS, their affinity to be stained with Trypan Blue solution 0.04%, their insertion behaviour into the artificial AC, the unfolding behaviour, pCEC-carrier transparency, and ability to adhere to the posterior stroma of the anterior remnant were rated on a 5-point scale (i.e. 0–5),

with higher score indicating greater similarity to *in vivo* DMEK/DSEK behaviour. *In vitro* surgeries with DMEK-grafts were used as a positive control and served as a reference for the scoring on the 5-point scale. All surgeries were performed by two DMEK surgeons (ID, IL).

For the *in vitro* surgery, main steps were performed as for standard DMEK surgery.[26] Three side ports were made at 2, 7, and 10 o'clock limbus, and the artificial AC was filled with PBS. Next, a 3-mm main incision was made at 12 o'clock to insert the pCEC-carrier constructs into the artificial AC. Each of the pCEC-carrier constructs was first stained for visualization with 0.04% Trypan Blue, implanted in the artificial anterior chamber and after unfolding and repositioning the pCEC-carrier constructs against the posterior stroma, the AC was filled 100% with an air bubble for 1 h to support the graft adherence to the stromal surface. Afterwards, air was partly removed through the paracentesis and attachment of the pCEC-carrier constructs to the stroma was evaluated by anterior-segment optical coherence tomography (AS-OCT; Heidelberg Engineering GmbH, Heidelberg, Germany).

RESULTS

Evaluation of cell viability and proliferation during *in vitro* expansion

The pCECs of a native porcine cornea formed a uniform monolayer of tightly packed, hexagonal cells (**Figure 1A**) with expression of the phenotypical markers ZO-1 and Na^+/K^+-ATPase (**Figure 1 B,C**). During *in vitro* cell expansion, the pCECs also displayed a uniform layer of tightly packed cells and showed expression of ZO-1 and Na^+/K^+-ATPase (**Figure 1 D–F**). Positive staining of Ki67 revealed that cultured pCECs retained the proliferative capacity (**Figure 2A**), while a low detection of nuclei of cells with compromised plasma membrane integrity (in green) indicated a high cell viability of the pCEC cultures ($96 \pm 4\%$) (**Figure 2B**) which is also reflected by the low degree of Trypan Blue staining (**Figure 1D**).

pCEC morphology after seeding on different carriers

The pCECs were able to retain endothelial morphology and to form a monolayer composed by closely packed cells when seeded onto LK20, LK100, and HALC (**Figure 3**). In addition, pCECs cultured on LK20, LK100, and HALC showed expression of the phenotypical markers ZO-1 (**Figure 4 A-C**) and Na^+/K^+-ATPase (**Figure 4 D-F**). Na^+/K^+ -ATPase expression had a more diffuse pattern all over the cell surfaces, whereas ZO-1 was mostly expressed on the cell borders.

In vitro surgeries with pCEC-carrier constructs

DMEK-grafts were used as a positive control for *in vitro* surgeries (**Figure 5**) and served as a reference for the scoring (**Table 1**). The pCEC-HALC constructs behaved very similar to a DMEK-graft (**Table 1, Figure 5**) and were implanted using a modified DMEK technique. These constructs naturally rolled on themselves into a double roll, but with the endothelium on the internal surface, unlike a DMEK-graft where the endothelium is on the external surface. Staining of the pCEC-HALC constructs with 0.04% Trypan Blue for visualization compared well to a DMEK-graft (**Table 1, Figure 5A**). The constructs could then be placed in a DMEK glass injector, with its correct orientation, i.e. curls

of the double roll facing downwards, checked under a surgical microscope. The pCEC-HALC carrier was implanted in a DMEK-like normal fashion; combined manoeuvres with air, fluid, and corneal indentation could be used in order to successfully unfold the tissue and position it against the posterior surface of the corneal stroma. Although special caution was needed in order to position the endothelium in its correct orientation due to its "inverse endothelium inside" location when compared with the regular DMEK graft, this was successfully achieved in all cases. One hour post-*in vitro* surgery, after partial air bubble removal, all constructs showed at least partial attachment resulting in an average score of 3.5 (on a scale of 0–5 with 5 resembling a DMEK).

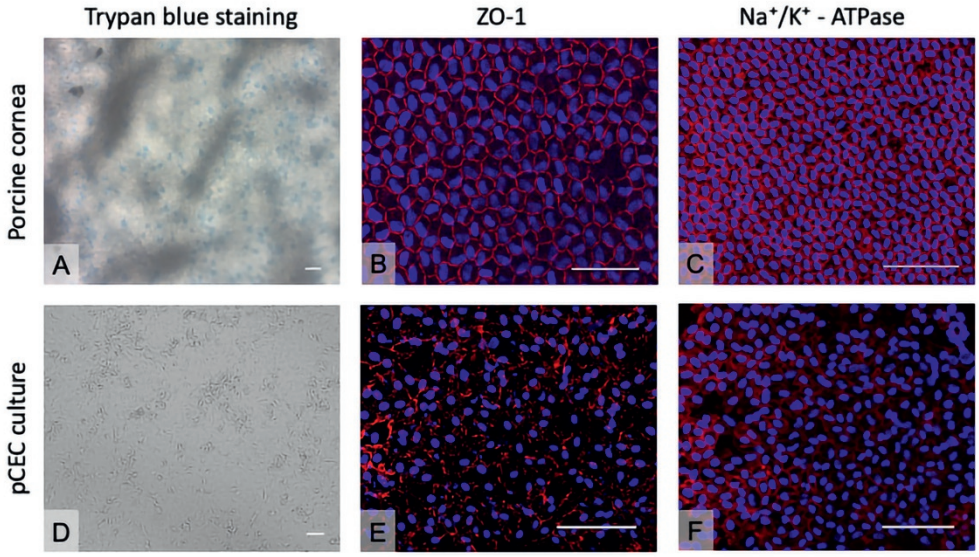

Figure 1: Evaluation of cell morphology on porcine corneas and of pCEC cultures. (A) Porcine cornea stained with hypotonic Trypan Blue solution 0.04%. (B) Expression of ZO-1 detected via immunofluorescence on porcine corneal endothelium. (C) Expression of Na^+/K^+-ATPase detected via immunofluorescence on porcine corneal endothelium. (D) pCECs cultured at P2 upon FNC-coated glass coverslips and stained with hypotonic Trypan Blue solution 0.04%. (E) Expression of ZO-1 detected via immunofluorescence on pCECs cultured at P2 upon FNC-coated glass coverslips. (F) Expression of Na^+/K^+-ATPase detected via immunofluorescence on pCECs cultured at P2 upon FNC-coated glass coverslips. Scale bars: 100 μm.

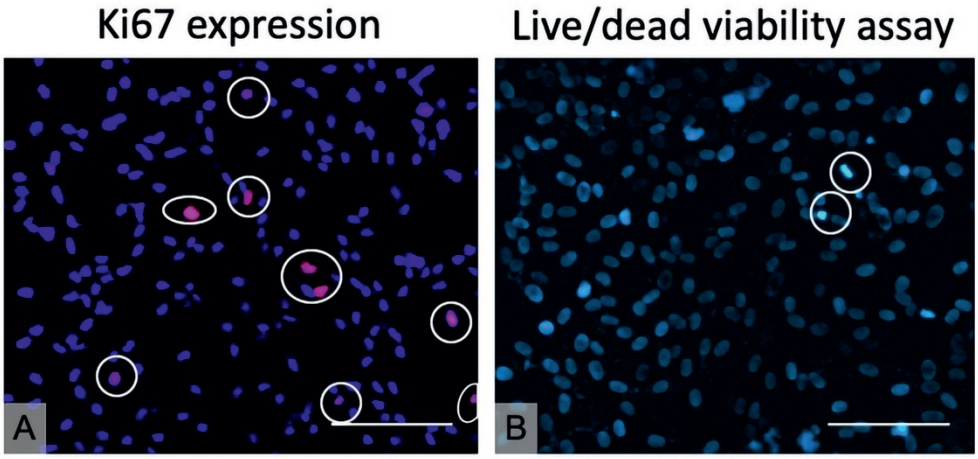

Figure 2: pCEC proliferation and viability at P2. (A) Evaluation of cell proliferation by expression of Ki67 (red, in white circles). Nuclei are stained by DAPI in blue. (B) Live-dead assay to determine cell viability (Blue: Live; Green, in circles: Dead). Scale bars: 100 μm.

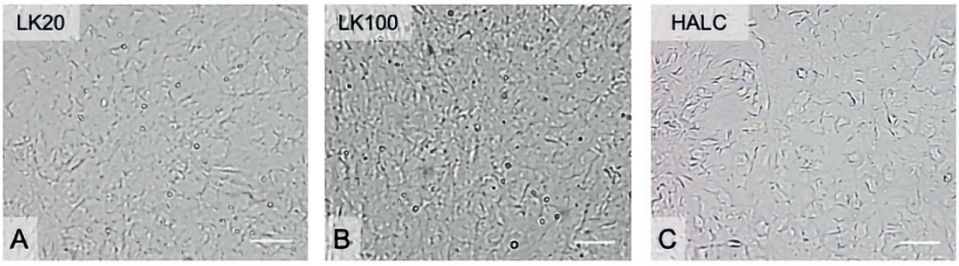

Figure 3: pCEC morphology at P2 on different biocompatible carriers. Series of illustrative figures showing successful pCEC cultures at P2 on chosen biocompatible membranes. (A) LK20, (B) LK100, (C) HALC. Scale bars: 100 μm.

The pCEC-LK20 constructs showed a Trypan Blue staining behaviour similar to the pCEC-HALC constructs and DMEK-grafts (**Table 1, Figure 5E**). These constructs had a tendency to form a large roll in BSS and could be loaded into the injector, but the "sticky" nature of the carrier prevented its easy insertion into the AC compared to a human DMEK-roll. Therefore, the pCEC-LK20 construct was folded over and inserted like a DSEK graft, a procedure in which the endothelial graft includes stromal tissue. Due to the increased thickness and

rigidity, the DSEK-graft does not roll but is folded like a "taco" for insertion (**Figure 5F**). Implantation in the AC and unfolding were more technically demanding compared to a DMEK-graft or the pCEC-HALC construct, as the pCEC-LK20 construct tended to be sticky, fragile, and hard to centralize due to its biomechanical properties (**Figure 5 F–G, Table 1**). Adherence to the bare stroma was also not optimal (**Figure 5H**), resulting in score of 2. Despite Trypan Blue staining (**Figure 5I**), pCEC-LK100 construct did not show any of the essential characteristics of a native DM (**Table 1**). Due to the thickness (100 µm) and increased rigidity of the carrier compared to normal DM, the construct was incapable to roll in BSS, and required a DSEK-like technique for implantation and unfolding (**Figure 5 J–K**) with insufficient adherence (**Figure 5L, Table 1**).

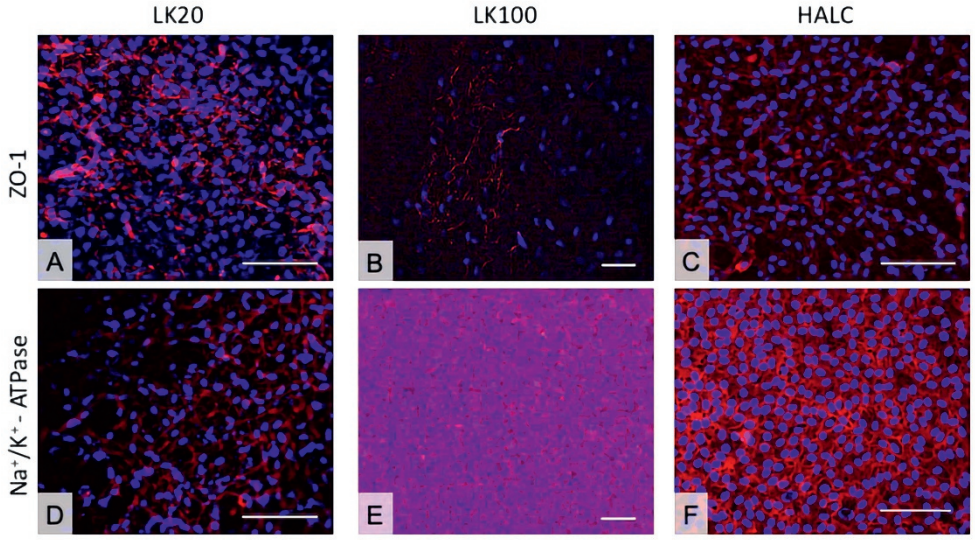

Figure 4: pCEC morphology check at P2 through immunofluorescence on different carriers. (A, B, C) ZO-1 expression detected in pCECs cultured on LK20, LK100, and HALC, respectively. (D, E, F) Na^+/K^+ expression detected in pCECs cultured on LK20, LK100, and HALC, respectively. Scale bars: 100 µm.

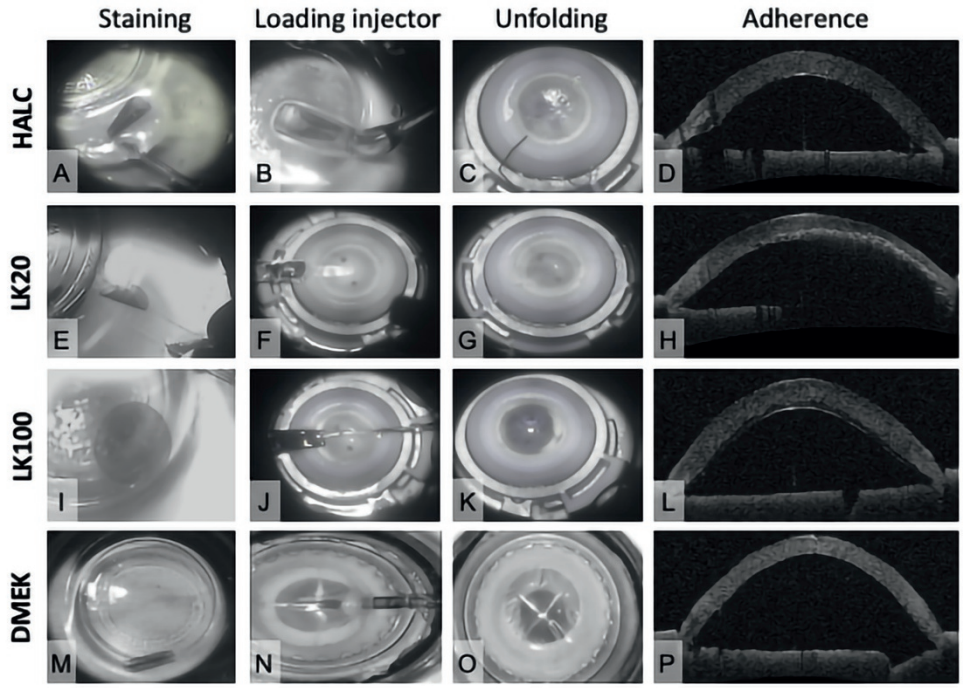

Figure 5: *In vitro* surgeries with pCEC-carrier constructs. The upper three rows show each of the three tested pCEC-carriers constructs after staining with hypotonic Trypan Blue solution 0.04% (A,E,I), insertion into the artificial chamber (B,F,J), unfolding (C,G,K), and adherence to the bare stroma visualized by anterior segment OCT measurement (D,H,L). The lower row (M–P) shows the corresponding images of a DMEK-graft that served as a positive control and reference point for the scoring.

Carrier type	DMEK-graft (positive control)	HALC	LK20	LK100
Rolling in BSS	5	4	1	0
Staining	5	3.5	3.5	5
Anterior chamber	5	4	2.5	1
Implantation		(similar to DMEK, easy orientation)	(lot of manipulation, sticky material)	(rigid material)
Protocol followed	DMEK	DMEK	DSEK	DSEK
Unfolding	5	4 (similar to DMEK, easy to centralize)	2.5 (fragile material, hard to centralize)	0
Transparency	5	4	3.5	0.5
Adherence	5	3.5	2	1

Table 1: *In vitro* surgeries with pCEC-carrier constructs: scoring scheme. Each parameter under evaluation was rated on a 5-point scale (i.e. 0–5) by two DMEK surgeons, with higher score indicating greater similarity to *in vivo* DMEK behaviour. A DMEK-graft was tested as a positive control and served as a reference point for the scoring. Values are reported as means.

DISCUSSION

In this study, we demonstrated that pCECs can be successfully cultured onto biocompatible, bioengineered collagen-based sheets, and onto HALC. Light microscopy and fluorescence microscopy analysis showed successful pCEC cultures up to P2 which retained their characteristic cell morphology, and distinctive expression of endothelial markers such as ZO-1 and Na^+/K^+-ATPase. However, upon *in vitro* transplantation, only the pCEC-HALC constructs behaved similar to DMEK-grafts in terms of staining, rolling in BSS, insertion in the anterior chamber, and attachment to the bare posterior stroma of a human donor corneo-scleral rim (**Figure 5**).

Endothelial keratoplasty is now the preferred treatment for corneal endothelial disease, but scarcity of human donor tissue led to alternative approaches such as *in vitro* expansion of CEC on suitable cell carriers and injection of cultured human CEC.[9,27–29] In previous studies, it was shown that cultivated monkey CEC cultured on a collagen type I carriers for 4 weeks produced a confluent monolayer expressing ZO-1 and Na^+/K^+-ATPase, and at 6 months after transplantation into monkeys, the cornea was still clear with a normal cell density.[30] In addition, hCEC have been shown to grow into a confluent layer on collagen type I-coated culture plates,[31] and cultured hCEC on collagen sheets composed of cross-linked collagen type I trans-planted in rabbits maintained 76–95% of pump function of human donor corneas.[11] Plastic compressed collagen type I, termed Real Architecture for 3D Tissues (RAFT), has been shown to support the growth of hCEC into a confluent mono-layer expressing ZO-1 and Na^+/K^+-ATPase.[32] However, substrates like RAFT have been reported to give rise to inflammation in experimental animal models. In addition, cultivation of CEC on biological carriers such as DM, HALC, and amniotic membrane has been reported.[16,17,28,33]

Ideally, the carrier should mimic DM in its biological and biomechanical characteristics, since this may create a microenvironment required for cellular activity and mechanical support during transplantation using the DMEK technique.[28] To that end, we selected carriers that differed regarding their artificial extra-cellular matrix (ECM) composition, technological process, and

biophysical (strength of cell-substrate adhesion or cell proliferation enhancement) and biomechanical properties (e.g. tensile strength, elasticity).

Our current study showed that pCECs cultured on the LinkCell™ collagen I-based sheets were able to retain endothelial morphology and to form a monolayer composed by closely packed cells with expression of ZO-1 and Na^+/K^+-ATPase. However, the behaviour of the pCEC-LK20 and pCEC-LK100 constructs during the *in vitro* DMEK surgery did not resemble that of a DMEK-graft since they had to be inserted in an artificial anterior chamber like a DSEK-graft (i.e. folded like a "taco") instead of rolled-up like a DMEK-graft.

Our study suggests that HALC may be one approach for use as a biocompatible carrier for CEC, though the need for human-derived tissues is not eliminated through its use and further *in vivo* studies need to be performed. HALC has several benefits as it resembles DM in terms of composition; the major component of HALC is the basement membrane protein collagen IV and other matrix component include collagen types I and III, laminin, fibronectin, and heparin sulphate proteoglycans.[34] The use of HALC as a scaffold for the cultivation of different ocular cells,[35–37] including hCEC,[17,32] has already been reported. It was shown that hCEC seeded on de-epithelialized HALC grew to confluency and strongly expressed ZO-1 and Na^+/K^+-ATPase. We confirm these results with our pCEC-HALC constructs and showed that they can be used successfully in *in vitro* surgeries.

In conclusion, LK20 carrier showed good biocompatibility, but required a DSEK-adapted surgical protocol, while *in vitro* DMEK surgery showed HALC as the most suitable carrier for cultivated pCECs.

DISCLOSURE STATEMENT

Dr. Melles is a consultant for DORC International/Dutch Ophthalmic USA and SurgiCube International. Dr. Dapena is a consultant for DORC International/Dutch Ophthalmic USA.

Dr. Rafat serves on the Board of Directors of the company LinkoCare Life Sciences AB, which is a spin-off firm developing products related to the research being reported, and holds relevant patents. Dr. Rafat's terms of arrangements have been reviewed and approved by Linköping University in accordance with its policy on objectivity in research. The other authors have no conflicting relationship to disclose.

ETHICAL STATEMENT

The study was carried out following the tenets of the declaration of Helsinki.

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