Biocompatibility of a fish scale-derived artificial cornea : cytotoxicity, cellular adhesion and phenotype, and in vivo immunogenicity

Accepted Manuscript

Biocompatibility of a fish scale-derived artificial cornea: cytotoxicity, cellular adhesion and phenotype, and *in vivo* immunogenicity

T.H. van Essen, MD, L. van Zijl, T. Possemiers, A.A. Mulder, S.J. Zwart, C.H. Chou, C.C. Lin, H.J. Lai, G.P.M. Luyten, M.J. Tassignon, N. Zakaria, A.El Ghalbzouri, M.J. Jager, MD, PhD

PII: S0142-9612(15)00911-4

DOI: 10.1016/j.biomaterials.2015.11.015

Reference: JBMT 17192

To appear in: *Biomaterials*

Received Date: 4 November 2015

Accepted Date: 6 November 2015

Please cite this article as: van Essen TH, van Zijl L, Possemiers T, Mulder AA, Zwart SJ, Chou CH, Lin CC, Lai HJ, Luyten GPM, Tassignon MJ, Zakaria N, Ghalbzouri AE, Jager MJ, Biocompatibility of a fish scale-derived artificial cornea: cytotoxicity, cellular adhesion and phenotype, and *in vivo* immunogenicity, *Biomaterials* (2015), doi: 10.1016/j.biomaterials.2015.11.015.

Biocompatibility of a fish scale-derived artificial cornea: cytotoxicity, cellular adhesion and phenotype, and *in vivo* immunogenicity

*T.H. van Essen[1], L. van Zijl[2], T. Possemiers[3], A.A. Mulder[4], S.J. Zwart[2], C.H. Chou[5], C.C. Lin[5], H.J. Lai[2], G.P.M. Luyten[1], M.J. Tassignon[3], N. Zakaria[3], A.El Ghalbzouri[4], M.J. Jager[1] **

1. Department of Ophthalmology, Leiden University Medical Center, Leiden, the Netherlands
2. Department of Research, Aeon Astron Europe B.V., Leiden, the Netherlands
3. Department of Ophthalmology, Antwerp University Hospital, Edegem, Belgium
4. Department of Dermatology, Leiden University Medical Center, Leiden, the Netherlands
5. Department of Research, Body Organ Biomedical Corporation, Tapei, Taiwan (R.O.C.)

Word count: 5372 (Body), 255 (Abstract)

Running headline*:* Biocompatibility of a fish scale-derived artificial cornea

Funding: This work was supported by a grant of Agentschap.nl. They had no involvement in study design, collection, analysis or interpretation of data, nor in the writing and decision to submit the paper.

Corresponding author (*):

M.J. Jager., MD, PhD

Department of Ophthalmology, J3-S

Leiden University Medical Center

PO Box 9600, 2300 RC Leiden

The Netherlands

Phone: +31-71-526 3097

E-mail: M.J.Jager@lumc.nl

Main author:

T.H. van Essen, MD

Department of Ophthalmology, J3-S

Leiden University Medical Center

PO Box 9600, 2300 RC Leiden

The Netherlands

Phone: +31-71-526 3938

Email: T.H.van_Essen@lumc.nl

Van Zijl, L

Department of Research,

Aeon Astron Europe B.V.,

J.H. Oortweg 19

2333 CH, Leiden, the Netherlands

Lisanne.van.zijl@aeonastron.com

Possemiers, T

Department of Ophthalmology

Antwerp University Hospital

Wilrijkstraat 10, 2650 Edegem, Belgium

Tine.Possemiers@uza.be

Mulder, A. A.

Department of Moleculair Cell-biology

Leiden University Medical Center

PO Box 9600, 2300 RC Leiden, the Netherlands

A.A.Mulder@lumc.nl

Zwart, S.J.

Department of Research,

Aeon Astron Europe B.V.,

J.H. Oortweg 19

2333 CH, Leiden, the Netherlands

Sarah.zwart@aeonastron.com

Chou, C. H.

Body Organ Biomedical Corporation

5F, No.153, Section 3, Xinyi Road

Da'an District, Taipei City 106, Taiwan (R.O.C.)

Charles.chou@aeonastron.com

Lin, C.C.

Body Organ Biomedical Corporation

5F, No.153, Section 3, Xinyi Road

Da'an District, Taipei City 106, Taiwan (R.O.C.)

Julio.Lin@aeonastron.com

Lai, H.J.

Aeon Astron Europe B.V.,

J.H. Oortweg 19, 2333 CH, Leiden, the Netherlands

Michael.Lai@aeonastron.com

Luyten, G.P.M.

Department of Ophthalmology, J3-S

Leiden University Medical Center

PO Box 9600, 2300 RC Leiden, the Netherlands

G.P.M.Luyten@lumc.nl

Tassignon, M.J.

Department of Ophthalmology

Antwerp University Hospital

Wilrijkstraat 10, 2650 Edegem, Belgium

Marie-Jose.Tassignon@uza.be

Zakaria, N.

Department of Ophthalmology

Antwerp University Hospital

Wilrijkstraat 10, 2650 Edegem, Belgium

Nadia.zakaria@gmail.com

El Ghalbzouri, A.

Department of Dermatology

Leiden University Medical Center

PO Box 9600, 2300 RC Leiden, the Netherlands

A.E.L.Ghalbzouri@lumc.nl

ABSTRACT

Purpose: To determine whether a fish scale-derived collagen matrix (FSCM) meets the basic criteria to serve as an artificial cornea, as determined with *in vitro* and *in vivo* tests.

Methods: Primary corneal epithelial and stromal cells were obtained from human donor corneas and used to examine the (in)direct cytotoxicity effects of the scaffold. Cytotoxicity was assessed by an MTT assay, while cellular proliferation, corneal cell phenotype and adhesion markers were assessed using an EdU-assay and immunofluorescence. For *in vivo*-testing, FSCMs were implanted subcutaneously in rats. Ologen® Collagen Matrices were used as controls. A second implant was implanted as an immunological challenge. The FSCM was implanted in a corneal pocket of seven New Zealand White rabbits, and compared to sham surgery.

Results: The FSCM was used as a scaffold to grow corneal epithelial and stromal cells, and displayed no cytotoxicity to these cells. Corneal epithelial cells displayed their normal phenotypical markers (CK3/12 and E-cadherin), as well as cell-matrix adhesion molecules: integrin-α6 and β4, laminin 332, and hemi-desmosomes. Corneal stromal cells similarly expressed adhesion molecules (integrin-α6 and β1). A subcutaneous implant of the FSCM in rats did not induce inflammation or sensitization; the response was comparable to the response against the Ologen® Collagen Matrix. Implantation of the FSCM in a corneal stromal pocket in rabbits led to a transparent cornea, healthy epithelium, and, on histology, hardly any infiltrating immune cells.

Conclusion: The FSCM allows excellent cell growth, is not immunogenic and is well-tolerated in the cornea, and thus meets the basic criteria to serve as a scaffold to reconstitute the cornea.

Keywords (4-6): artificial cornea, keratoprosthesis, collagen, in vitro, in vivo, biocompatiblity

INTRODUCTION

Of the 39 million people worldwide who suffer from corneal blindness, approximately 12% are affected bilaterally, with the developing countries having the highest percentages of corneal blindness.[1-3] Corneal blindness may be caused by corneal opacities, trachoma, and childhood blindness.[3]

There are multiple barriers that prevent restoration of vision in these patients through corneal transplantation, and these problems are especially common in developing countries: lack of access to drugs such as steroids and antibiotics, and lack of trained surgeons, but the most important one is lack of corneal donor tissue and the availability of those corneas at an affordable price.[3, 4] Even in the developed countries the cost of a donor cornea remains high, for example the cost of a donor cornea in the United States is approximately 3000 US dollars.[5]

The shortage of donor corneas may be reduced by using keratoprostheses as artificial corneas, as these keratoprostheses, such as the Boston Keratoprosthesis (B-Kpro) and Osteo-Odonto-Keratoprosthesis (OOKP), are viable solutions.[6, 7] However, due to potential complications and high surgical demands, such implants are currently only used in high risk patients. Furthermore, the B-Kpro still has the risk of rejection as it uses a donor cornea, while the OOKP has a complex implantation procedure, and both are rather expensive, although the developers of the B-Kpro are working towards cost reduction.[8] Three-dimensional scaffolds made from biomaterial, mimicking the corneal stroma, may be repopulated by the patient's own corneal cells, and those cells may in turn regenerate corneal tissue.[9-11] The use of biomaterials would reduce the chance of immune rejection. Three-dimensional scaffolds can be fabricated,[10] or harvested in an almost ready state from, for example, porcine corneas, [12] or a porcine cornea may be modified before use.[13]

Different biomaterials can be used and indeed are being explored as the basis for scaffolds.[4, 14-17]

The human corneal stroma consists primarily of type I collagen, arranged in orthogonal lamellae, which give the cornea its enormous tensile strength. [18] This makes collagen a logical material for use as an artificial corneal scaffold.[15, 19] Indeed, one synthesized collagen (recombinant human

collagen type III) scaffold has reached the clinical phase I, but a drawback is the complicated fabrication process;[20] another one is achieving enough tensile strength of the construct to allow interrupted or continuous suturing.

Decellularized human corneas can also be regarded as collagen-based scaffolds but use of these scaffolds will not decrease the lack of need for tissues. Decellularized porcine corneal stroma is another option, but this confers a risk of transmitting animal disease.[15] Screening for pathogens may reduce that risk.[15]

Another source for three-dimensional collagen scaffolds are fish scales.[21-23] Scales from the Tilapia fish, farmed under controlled conditions,[24] are composed of collagen type I, arranged in a similar way as in human corneas.[25] Such scaffolds may offer a cheap alternative and facilitate corneal regeneration.

Previous studies showed that a collagen scaffold, made of the scales of the Tilapia fish (Oreochromis niloticas) by decellularization and decalcification, facilitates repopulation by rabbit corneal stromal cells and has a high oxygen permeability.[23] Our previous experiments showed that this scaffold has adequate light transmission values and is suitable for use in keratoplasty, using intermittent sutures.[26]

As we hypothesized that the fish scale-derived collagen matrix (FSCM) can be used as an implant for corneal reconstruction, we set out to perform an in depth investigation into the suitability of this implant for corneal reconstruction. We measured the tensile strength and glucose permeability of the FSCM-derived scaffolds, cultured primary human corneal epithelial and stromal cells together with the scaffold to assess the scaffold's cytotoxicity, and determined its effect on the proliferation, phenotype and adhesion of corneal cells *in vitro*; finally, we studied the body's response upon subcutaneous and corneal intrastromal placement of the FSCM in rats and rabbits, respectively.

METHODS

Fish-scale derived collagen matrix

The collagen matrices were prepared from the scales of Tilapia (Oreochromis Niloticas), bred under controlled conditions as previously described.[23, 24, 26] Decellularized, decalcified and gamma-irradiated FSCM were provided by Aeon Astron Europe BV (Leiden, the Netherlands) and stored in sterilized phosphate-buffered saline (PBS) containing 1% penicillin/streptomycin (15140-122; Gibco by Life Technologies, Bleiswijk, the Netherlands) at 4° Celsius until use. The matrices were 8.0 ± 1.0 mm in diameter, 0.25 ± 0.05 mm in thickness, and had no curvature, except the ones used for the rabbit experiment.

Tensile strength and glucose permeability

The tensile strength of the matrices was determined using a universal testing machine (Instron 3365, UK) with a gauge length of 10 mm. The test was performed at a crosshead speed of 6mm/min at room temperature. Measurements were performed on four independent samples.

Glucose permeability was assessed with the USP <1724> Vertical Diffusion Cell Test using the Franz cell system. Matrices (n=4) were fixed between a donor chamber containing 50mg/ml glucose solution and a receptor chamber with distilled water. After 1, 6, and 18 hours, water from the receptor chamber was removed for glucose measurement, for which a glucose assay kit was used (Cat no. GAGO20-1KT; Sigma-Aldrich, Diegem, Belgium).

Primary human corneal cells

Human cadaveric eyes were obtained from the EuroCorneabank, Beverwijk, the Netherlands. Corneal epithelial cells were harvested by cutting the cornea into four equal quadrants, washing the corneal pieces three times in PBS (phosphate-buffered saline) and incubating the tissue overnight at 4°C in 2.4 U/ml dispase II (04 942 078 001; Roche Applied Science, Mannheim, Germany) in PBS. The corneal epithelium was manually separated as a sheet from the underlying tissue by gentle use of

forceps and a spatula, centrifuged (5 minutes at 1000 rpm), and incubated in 80 µl TrypLE (TrypLE Select 126050-10; Life Technologies Europe BV, Bleiswijk, the Netherlands) for 10-15 minutes at 37°C to create a single cell suspension, which was cultured in CnT-20 medium (CnT-20; Bio-connect BV, Huissen, the Netherlands) with 1% penicillin/streptomycin.

The cornea was cut into small pieces of about 1 x 1 mm to isolate corneal stromal cells. The corneal parts were placed in a 0.1% collagenase type II solution (17101-015; Life Technologies Europe BV) and incubated overnight at 37°C. The obtained cell solution was put through a 70 µm cell strainer, subsequently centrifuged (5 minutes at 850 rpm) and cultured in DMEM/HAM F12 medium with stable glutamin (FG4815; Biochrom AG, Berlin, Germany), supplemented with 5% fetal calf serum (758093; Greiner Bio-one GmbH, Frickenhausen, Germany) and 1% penicillin/streptomycin.

Cytotoxicity and proliferation

Cytotoxicity was analyzed using an MTT viability assay, where epithelial and stromal cells were directly and indirectly exposed to the FSCM (P12261201/P12041201) for 24h. In order to determine cytotoxicity through direct cell contact, the cells were seeded and the scaffold was added to the culture. To assess any indirect cytotoxicity, the cells were cultured in the presence of FSCM extract (cell culture medium that had been incubated with the scaffold for 24h at 37°C) and a dilution series of the extract (1:2 and 1:4). As a positive control, cells were treated with 1% sodium dodecyl sulfate, while untreated cells served as a negative control. For the MTT assay, 1 mg/ml 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) (M2128-5G, lot: 046K5304; Sigma-Aldrich, Diegem, Belgium) was added to the cell cultures and the cells were incubated for 2 hours at 37°C. Metabolically active cells reduce MTT to formazan and after formazan extraction using isopropanol, the optical density was measured using a spectrophotometer (at 570nm). This assay was performed in triplicate.

To assess proliferation, the two-donor mixes of epithelial (40,000 cells) and two-donor mixes of stromal cells (25,000 cells) were seeded onto the smooth side of the FSCM (P12261201) and on

plastic discs, which served as controls. Experiments were carried out in duplicates. After 24, 48, and 72 hours, cells were incubated with 1% of 5-ethynyl-2'-deoxyuridine (EdU) (Click-It® EdU Alexa Fluor® 594 Imaging Kit; C10339; Life Technologies Europe BV) for 16 hours, and, after being washed with PBS, cells were fixed with 4% formaldehyde in PBS, and permeabilized by 0.5% Triton® X-100 EdU was visualized using Alexa Fluor® 594. DAPI counterstaining served to identify cell nuclei. EdU-positive cells, indicating actively dividing cells, were manually counted by two different observers in three randomly-selected areas on the FSCM, using a confocal microscope.

Immunofluorescence, SEM and TEM

Corneal epithelial cells were seeded on the patterned side of four FSCMs (15,000-50,000 cells/FSCM), and on the smooth side of two FSCMs (P06271201), and cultured until sub-confluence (11-12 days), at which time the FSCMs were prepared for whole mount immunofluorescence to assess cell phenotype and adhesion. For this, the scaffolds were fixed in 4% formaldehyde, and antibodies were applied against CK3/12 (1:50; bs-2369R-Cy7; Bio-Connect BV, Huissen, The Netherlands), a marker of differentiated corneal epithelium, and the cell-matrix adhesion molecules integrin α6 (1:1000; ab20142; Abcam; Cambridge, UK) and β4 (1:1000; ab110167; Abcam; Cambridge, UK). After incubation with fluorochrome-labeled secondary antibodies, counterstaining was performed with DAPI (cell nuclei) and Alexa Fluor 488 Phalloidin (F-actin filaments) (1:100; A12379; Life Technologies Europe BV; Bleiswijk, the Netherlands).

The experiments with the corneal epithelial cells were repeated at another location (University of Antwerp, Antwerp, Belgium) using corneal limbal explants from one human donor cornea. Each limbal explant was placed on top of four different non-curved FSCMs (P12261201), and additionally on top of two dome-shaped FSCMs (P08081304; diameter curvature 7.4 ± 0.2 mm) and cultured in Corneal Epithelial Culture Medium[27] (supplemented with collagen and fibronectin) for 22 days for immunofluorescence and 20 days for analysis with Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM). Whole mount immunofluorescence was performed on the four

non-curved FSCMs, that were cut in halves, for CK3/12 (1:100; ab68260; Abcam, Cambridge, UK), E-cadherin (1:400; ab53033), integrin α6 (1:1000; ab20142), laminin-332 (1:25; ab11575; Abcam; Cambridge, UK), and additionally with ΔN P63 (1:300; sc-8609; Santa Cruz Biotechnology; CA, USA), a determinant for stemness. Corneal stromal cells were also cultured on the smooth side of FSCMs (P12261201), coated with and without rat tail collagen type I. The cells were stained for whole mount immunofluorescence with antibodies against the transmembrane receptors integrin-α6 (1:1000; ab20142) and integrin-β1 (1:50; ab52971; Abcam, Cambridge, UK), involved in cell-matrix adhesion, and counterstained for F-actin (Alexa Fluor 488 Phalloidin; A12379) to depict the cytoskeleton, and with DAPI for cell nuclei.

Post-modifications

FSCMs (P04201201)[23, 24, 26] were treated with femto second laser to create FSCMs with vertical non-penetrating pores only, or in combination with 1x890 µm (H x W) horizontal tunnels. Additionally, two scaffolds were incubated for 3 hours at 37°C in PBS with 4.8 U/ml dispase II to detach the collagen layers. Corneal stromal cells were cultured on these manipulated FSCMs (200,000 cells/FSCM). When complete confluence over the FSCM (2-3 weeks) was obtained, the samples were fixated in 4% formaldehyde and hematoxylin-eosin (HE) staining and immunofluorescence with F-actin and DAPI was performed to visualize the effect on the FSCMs and on the behavior of the cells.

Subcutaneous implantation

Forty male Fischer 344 albino rats (Charles River Laboratories, France), weighing between 280-336 grams, were used for subcutaneous implantation of the FSCM. Ethical approval was given by the Animal Experiments Committee of the Leiden University Medical Center (Leiden, the Netherlands), and all animals were treated according to the ARVO statement for use of animals in ophthalmic and vision research. Animals were given at least one week for acclimatization.

Prophylactic pain reduction was started pre-operatively with acetaminophen infusion solution (50mg/ml) added to the drinking water at 1 mg/ml, which was continued during seven days. The rats were anesthetized with isoflurane and a subcutaneous injection of 50 µl bupivacaine (5mg/ml) at the site of implantation. A 2 cm long incision was made in the right, high dorsal flank, where a subcutaneous space was created, and the matrix inserted. Twenty animals received the FSCM (P09151101) and twenty other animals another matrix, the Ologen® Collagen Matrix (Aeon Astron Europe, Leiden, The Netherlands; 12 mm in diameter, 1 mm in thickness; porcine collagen type I) as a control; Ologen® Collagen Matrix is used for bleb filtration in trabeculectomy surgery in humans.[28] All matrices were cut in half and soaked in antibiotics for 5 min (Polyspectran® containing Gramicidine, neomycine and polymyxine B) prior to insertion. The incision was closed with 6/0 absorbable sutures (Safil REF 1048734, B. Braun Medical BV, Oss, The Netherlands). Seven animals of the twenty with an FSCM, and eight animals of the twenty with an Ologen® implant, additionally underwent sham surgery on the contralateral dorsal flank, which served as a control (n=15) to determine whether the surgery itself influenced local inflammation in the rat subcutaneous tissue. At 1, 6, and 11 weeks post implantation, five animals with an FSCM and five with an Ologen® implant were sacrificed. Three weeks after the first implant, a second FSCM was implanted in the contralateral dorsal flank of five animals of the FSCM group and a second Ologen® implant in five animals of the Ologen® group to study immune sensitization; these animals were sacrificed one week after the second implant.

After the animals were sacrificed, the subcutaneous tissues were harvested, formalin fixed and embedded in paraffin. Sections were cut and stained with HE. Based on the HE staining, three representative cases were selected out of the five per group per time point, which were additionally stained with antibodies to identify granulocytes (MPO staining; 1:1000; ab45977; Abcam), macrophages (ED-1 staining; 1:300; MAB1435; Millipore BV, Amsterdam, The Netherlands), T cells (CD3 staining; 1:500; MCA772GA; Sanbio BV, Uden, The Netherlands), and immunoglobulins

(Polyclonal Rabbit Anti-Rat Immunoglobulins/HRP, mainly IgG; 1:100; P0162; Dako BV, Heverlee, Belgium).

For automatic scoring with a computer program (ImageJ;[29] W.S. Rasband, ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2014), three images were made of two subsequent sections per matrix at standard locations (left end, middle, and right end of the matrices), and the percentage positively-staining area per view was calculated for each staining. An independent pathologist graded the amount of immune response as: minimal, mild, moderate, and severe, based on the HE staining.

Intrastromal implantation

Fourteen female New Zealand White rabbits (Charles River Laboratories, France), ranging from 2 – 3 kilograms, underwent intrastromal surgery, with seven animals receiving a curved FSCM (P08081304; 120 ± 20 µm in thickness, curvature 7.4 ± 0.2 mm in diameter) in their right eye, while seven other animals received no implant (while undergoing the same surgical procedure), serving as controls. Ethical approval was given by the Animal Experimentation Committee of the University of Utrecht (Utrecht, the Netherlands) where the experiment was conducted, and all animals were treated according to the ARVO statement for use of animals in ophthalmic and vision research. All rabbits underwent at least one week of acclimatization.

Prophylactic painkilling was started preoperatively with a subcutaneous injection of Carprofen (4-5mg/kg) and this was given postoperatively once daily for six days. The anesthetic regimen consisted of intramuscular injections of Ketanest-S® (ketamine 15 mg/kg/h) and Dexdomitor® (dexmedetomidine 0.125 mg/kg/h), and local administration of one drop oxybuprocaine 0.4% every 15 minutes.

A corneal incision was made with a precision depth knife set at approximately 80% of the corneal thickness, and the corneal layers were dissected horizontally with a blunt spatula until a pocket was formed of at least 4 mm width and 5 mm length. An FSCM was trephined to 3.8 mm and inserted

into this corneal pocket, and the incision was closed with 10/0 nylon sutures. Sutures were removed at 3.5 weeks follow-up.

The eyes were treated postoperatively with tobramycin/dexamethasone eye drops twice daily for eleven days. When symptoms of ocular irritation arose, tobramycin/dexamethason treatment was restarted for all animals, together with artificial tears (Visidic Carbogel; Tramedico BV, Weesp, the Netherlands), until the symptoms subsided, after which the treatment was reduced, but continued until euthanasia after 6 weeks of follow-up.

Eyes were checked with a surgical microscope at 1, 2, 3.5, and 6 weeks for conjunctival hyperemia, corneal haze and neovascularization, and ocular discharge. At 6 weeks, the eyes were additionally observed through a slit-lamp, and the animals were euthanized. The corneas were harvested, fixated in formaldehyde and embedded in paraffin. HE staining was performed as well as an immunohistochemical staining with an antibody against RAM11 (1:25; M063301; Dako BV) to identify macrophages.

Long-term intra-stromal implantation

Two other New Zealand White rabbits were used used for long-term follow-up. In these animals a partial depth corneal incision was made to create an intrastromal pocket of 6.5 mm in diameter using a crescent 2.0mm knife. An FSCM was trephined down to 6 mm in diameter and inserted to the pocket. The incision opening was closed with 10-0 nylon sutures. Postoperatively daily drops of Levofloxacin 0.5% and Prednisolone acetate 1% (Pred Forte) were administred during the first 4 weeks. Follow up was performed at week 1, 2, 3, 4 and 54 with optical coherence tomography (OCT) (iVue100, Optovue Inc.).

RESULTS

Tensile strength and glucose permeability

Four FSCM's were used to measure the tensile strength and glucose permeability. The Young's modulus for tensile strength was calculated from the stress-strain curve and was 11.7±0.92 MPa. The glucose diffusion flux was maximal after 1 hour (24.9± 6.8 x 10^{-6} cm^2/sec). The diffusion flux decreased to 6.0±0.5 and 2. 6±0.3 x 10^{-6} cm^2/sec at 6 and 18 hours.

Cytotoxicity

Human corneal epithelial and stromal cells were co-cultured with the FSCM to test for any cytotoxic effects using an MTT assay. Cell viability was assessed and compared to unexposed controls (Figure 1). For the direct contact test, an FSCM was added to the culture, while for the indirect contact test, the cells were cultured in the presence of FSCM extract (cell culture medium that had been incubated with the scaffold for 24h at 37°C) and a dilution series of the supernatant was tested. Using two FSCM, corneal epithelial and stromal cell viability in the direct contact test was 79.6% and 83.4%, and for the indirect contact test 83.7% and 89.3%. This was higher than the threshold for cytotoxicity, which has been set at 70% according to the ISO-standard (ISO: 10993-5:2009(9)).

Proliferation

Next, we determined whether the FSCM influenced cell proliferation. Human corneal epithelial and stromal cells were seeded on an FSCM and on plastic, and dividing cells were counted after 24, 48, and 72h of culture using EdU staining. The percentage of actively-dividing (EdU positive) epithelial or stromal cells on the FSCM was similar to the number of EdU-positive epithelial or stromal cells cultured on plastic discs (Figure 2A and 2B).

Phenotype and adhesion

After having assessed cytotoxicity and cell proliferation, we determined if there might also be an effect on morphogenesis or on adhesion molecules. Therefore, primary human corneal epithelial cells were cultured on FSCMs and assessed using immunofluorescence for the presence of different markers. The cells proliferated on both the anterior as well as the posterior side of the FSCM, and we observed no difference in cell growth with regard to the different micropatterns present on the FSCM (spokes, ridges, and spikes on the anterior side, and no pattern (smooth) on the posterior side (Figure 3)). The seemingly black, unpopulated areas (Figure 3B) were out of focus: changing the focus of the confocal microscope showed that these areas were populated as well.

Primary human corneal epithelial cells were grown until confluence on the FSCM in two different laboratories and assessed for phenotype and adhesion markers with immunofluorescence staining (Figure 4A). The epithelial cells were CK3/12-positive, and displayed cell-cell adhesion and cell-matrix adhesion molecules (E-cadherin, integrin-α6, integrin-β4, and laminin 332). The primary human stromal cells, the keratocytes, cultured on the FSCM, were positive for integrin-α6, and integrin-β1 (Figure 4B). No difference in expression was observed when FSCM's were coated with rat tail collagen type 1 (data not shown).

The TEM images revealed that stratified primary epithelial cells grown on top of the FSCM developed a stratified layer, which had approximately six cell layers, and that desmosomes were present between the epithelial cells, while hemidesmosomes were seen between the basal epithelial cells and the underlying FSCM (Figure 4C).

Post-modifications to stimulate cellular ingrowth

We wondered whether it was possible to post-modify the FSCM to stimulate cell ingrowth into the scaffold, and to test this, the FSCM was incubated in dispase and modified by femto-second laser (Figure 5A). Incubating the FSCM in 2.4 U/ml dispase II for 4h resulted in in a space between the dense collagen layers at the border-area of the matrix (Figure 5B). We additionally created stromal pores using a femto-second laser and cultured primary corneal stromal cells on top of this modified

FSCM. Confocal immunofluorescence images depicted cells that filled up the pore of the FSCM, as well as the small spaces created between the dense collagen layers (Figure 5C). When a FSCM was used that had been modified with pores and horizontal tunnels of approximately 1 micrometer in height, corneal stromal cells were found to protrude their cytoskeleton into those tunnels (Figure 5D).

Immunogenicity

To determine the immunogenicity of the FSCM, a classical challenge test was performed in rats. The FSCM was implanted subcutaneously in the dorsal flank of rats (n = 20) and the tissue response was compared to a matrix used in human bleb filtration for trabeculectomy (n = 20), as well as to sham surgery (n = 15). The tissue response was homogenous in each group, although some variations in amount and composition of infiltrating cells existed and one animal, at the end point, showed fibrous capsule formation. Figure 6A shows the HE staining of the FSCM and surrounding subcutaneous tissue at 11 weeks. Immunostaining for granulocytes, macrophages, T cells, and immunoglobulins showed more variation at 1 week postoperatively than after 6 and 11 weeks postoperatively. The composition of infiltrating immune cells one week after implantation was dominated by granulocytes, but also contained macrophages in all groups (Figure 6B). The immune response at 1 week was graded as moderate and was not significantly different from the two control groups (Figure 6C). The immune response diminished to mild and minimal over time until the end point at 11 weeks, for each of the four immunomarkers, and the response to the FSCM was again not different from that seen with the control matrix (Ologen® Collagen Matrix), and sham surgery (Figure 6C). Microscopic analyses revealed that the increased amount of macrophages present in the FSCM group at 1 week, and of granulocytes at 11 weeks, were due to false-positive counting of the folds on the sections for which the ImageJ software could not correct, thereby creating high error-bars. These samples were not excluded from the analysis.

A subcutaneous immunological challenge with an FSCM, three weeks after the first exposure to the FSCM, elicited no increased immune response after 1 week (Figure 6D).

Corneal compatibility

Finally, we evaluated the *in vivo* compatibility of the FSCM: curved FSCMs were implanted intrastromally in rabbit corneas to assess the corneal tissue response to the FSCMs. Rabbits that underwent sham surgery served as controls. The animals were observed for six weeks, and the corneas were subsequently analyzed histologically. Sutures were removed after 3.5 weeks as they gave rise to ocular irritation in both groups, which disappeared upon removal of the sutures. The cornea stayed clear throughout the follow up, and no epithelial defects or signs of melting were observed (Figure 7A). Some corneal vascularization was present in the superior limbal area in both groups, but this was similar to the situation in the non-operated contralateral eyes (Figure 7B). The corneal stroma showed a normal arrangement of its fibers around the FSCM. A few infiltrating cells were noticed around the edge of the FSCM (Figure 7C). The infiltrate contained a mix of corneal stromal cells (keratocytes) and macrophages (Figure 7D and 7E). No granulocytes were seen throughout the sections and in a few sections, a single T cell was observed. The activated cells were not seen to invade the FSCM, but appeared to deposit new extracellular matrix at the edges (Figure 7D and 7E). The FSCM slightly pushed up the overlaying corneal stroma, resulting in a slight atrophy of the otherwise healthy epithelium covering the area positioned above some of the more superficially-implanted FSCMs, while showing normal epithelial morphology in the ones with deeper implants which caused less rising of the anterior stroma (Figure 7C).

The two rabbits implanted intrastromally with 6 mm diameter FSCM's for long-term followup revealed no significant immune response as based on the in vivo follow up and the OCT images. The OCT revealed that there were no signs of extrusion of the implanted FSCM and that the thickness of the FSCM remained stable (Figure 8).

DISCUSSION

Our data show that the FSCM is suitable for use as basic material to develop an artificial cornea. It facilitates adequate epithelial regrowth and adhesion, is not immunogenic, and can be stably implanted in the cornea. 11.7±0.9 MPa.

The glucose diffusion flux was maximal after 1 hour (24.89± 6.78 x 10^{-6} cm^2/sec).

The glucose diffusion rate of the FSCM (24.9± 6.8 x 10^{-6} cm^2/sec) is more than adequate, when compared to the human cornea (2.5-3.0 x 10^{-6} cm^2/sec).[30] The tensile strength (11.7±0.9 MPa) is also nearly identical to that of the human cornea (3-9 MPa).[31] It is of paramount that any material used as a basis to develop an artificial cornea has no unwanted effects that interfere with its purpose: to replace a damaged cornea for a given period of time. The material should be non-toxic to surrounding cells and not have a major influence on a cell's proliferative capacity. Our data illustrate clearly that the FSCM is neither cytotoxic nor has a major influence on epithelial proliferation. As the FSCM is being developed as a stromal replacement device, it should facilitate regrowth and adhesion of stable and healthy corneal epithelium. Our *in vitro* studies show that primary human corneal epithelial cells that grow on the FSCM are of the correct phenotype, i.e. expressing CK3/12, E-cadherin and desmosomes, the latter of which are both involved in cell-cell contact and communication between epithelial cells). Intact cell-cell contact and communication is very important in epithelial tissues to fulfill their barrier function.[32, 33]

The presence of hemi-desmosomes and one of its components, the heterodimer integrin-α6 and integrin-β4,[34, 35] shows that these cell-matrix adhesion molecules are produced by the epithelial cells, and indicates that cell-matrix adhesion is indeed present; this is essential for firm adhesion of newly-formed corneal epithelium to the FSCM. We also show the presence of laminin-322, as integrin-α6β4 preferentially binds to this protein, which is produced by epithelial cells and is part of the basal membrane, and strengthens the connection with the underlying collagen.[36] The finding that primary human epithelial cells are able to stratify when grown in specific medium on top of the FSCM greatly supports the use of this scaffold as a replacement therapy for corneal stroma.

Once the FSCM is implanted in the cornea, its border is adherent to the surrounding corneal stromal tissue. When the FSCM is sutured into place, the sutures should provide enough strength to hold it in situ. For long term use, the FSCM should become attached to the surrounding recipient's corneal tissue. In this adhesion process, the corneal stromal cells play a pivotal role, and our *in vitro* studies with primary corneal keratocytes show that they grow on the FSCM and express the cell-matrix adhesion molecules needed by keratocytes (Figure 4B): integrins α6 and β1[37] .

In order to improve the physical interaction between the FSCM and the keratocytes, the FSCM can be modified using several techniques. We manipulated the FSCM with a femto second laser and dispase II, which led to additional attachment points for keratocytes. Additionally, cross-linking may be applied, a technique used to produce the curved FSCM, which was used for intrastromal implantation in rabbit corneas. That this FSCM is suited for post modification makes it ideal to adjust it as needed during its development towards an artificial cornea.

Implantation of the FSCM subcutaneously in rats allowed us to compare the immune response against the FSCM with the response against a collagen matrix that is already safely used in humans as well aswith sham surgery: we demonstrated that the FSCM is not immunogenic, as there was no specific immune response, and there were no signs of sensitization upon reintroducing the FSCM subcutaneously in the same animal. This confirms our hypothesis as posed in our previous work,[26] namely that the inflammation following corneal placement in a pocket or as an anterior laminar graft was largely due to mechanical irritation. The loose rat skin still allowed some shifting of the FSCM, which is denser and harder than the spongy Ologen® Collagen Matrix, and in one case elicited fibrous encapsulation. Despite the possibility of movement of the matrix, and despite that the skin is vascularized and not immunosuppressed, only a few immune cells and no specific immune responses were observed.

Our focus in the rabbit experiment, with intrastromal implantation of the FSCM, was to test whether the FSCM could have a negative effect on the cornea, i.e. melting of the overlying epithelium or eliciting an unwanted immune response during a longer follow up period (six instead of three

weeks). For that reason we implanted a thinner and curved FSCM, thereby minimizing mechanical irritation. We used intrastromal implantation instead of an anterior lamellar keratoplasty , as we intended to determine the potential response elicited by the FSCM, and not by sutures or mechanical irritation. Using this model, we were able toplace the sutures far away from the FSCM, which helped in distinguishing between a response elicited by and towards the sutures versus one elicited by the FSCM. We observed that all corneas stayed transparent, the overlaying epithelium remained healthy and stable during 6 weeks of follow-up, and on histology, a few infiltrating macrophages were observed; we can thus conclude that there were no unwanted adverse effects. Keratocytes were located near the skirt of the FSCM and appeared to interact with the exposed edges and wehypothesize that these keratocytes may be slowly replacing the FSCM's collagen type I by extra-cellular matrix deposition. The observed empty spaces between the layers at the FSCM's edge are considered to be due to the trephination. Long-term follow-up suggests that the FSCM will remain stable without extrusion, degradation or rejection for at least up to one year. Some scarring around the FSCM was visible however, which is something we should address.

When looking into the literature, there are several other solutions for corneal restoration. In contrast with the Boston Keratoprostheses, the FSCM does not need a donor cornea. [38] The OOKP is a complex but well researched solution as all other options for corneal restoration have failed,[39] , and the FSCM may offer an alternative.

A synthetic transparent scaffold (ACTO TexKPRO; polyvinylidene difluoride fibers and silicone) has successfully been implanted as preliminary treatment in six patients for 6 to 40 months after which a keratoplasty was performed. This ACTO TexKPRO is currently being developed as a solution for cases where the ocular surface has been destroyed.[40] A recent publication evaluated a hydrogel (polyethylene glycol/polyacrylic acid) designed as artificial cornea, also by intrastromal implantation in rabbits.[41] Fifty percent of the rabbits followed for 2 months (n=6) kept clear corneas without inflammation. However, after up to 16 months follow up, there was a high rate of complications, such as epithelial defects, corneal thinning and even extrusion. Yet in a previous study, this hydrogel

showed clear corneas up to around 6 months. [42] Our two rabbits had no extrusion or notable corneal thinning at 1 year follow up.

A clinically-used biosynthetic artificial cornea that is made from carbodiimide cross-linked recombinant human collagen, has been shown to be stable for up to 4 years of follow-up in 10 patients, although it resulted in thinner corneas (358 ±101 µm).[16] This material is quite costly, and seems to be the closest to our material.

We showed previously that the FSCM has identical direct light transmission as the human cornea (although its light scatter values can be further optimized), and that it has a low immunogenicicty. In our current study, we show that the FSCM is a scaffold that has no adverse effects to corneal cells *in vitro*, or to the cornea *in vivo* in the short term, and most likely also not in the long term. This clears the road for initiating larger longer follow-up experiments and to optimize its suturability. Further work will focus on the use of the FSCM as a corneal replacement strategy when no human material is available, and on the suitability of the FSCM as an emergency solution.

Acknowledgements

We thank Veerle van Gerwin of the Department of Ophthalmology at the Antwerp University Hospital, in Belgium, for the much appreciated help with the optimaliations steps for the immunofluorescence.

REFERENCES

1. Resnikoff S, Pascolini D, Etya'ale D, et al. Global data on visual impairment in the year 2002. *Bull World Health Organ*. 2004;82:844-851.

2. Pascolini D, Mariotti SP. Global estimates of visual impairment: 2010. *Br J Ophthalmol*. 2012;96:614-618.

3. Oliva MS, Schottman T, Gulati M. Turning the tide of corneal blindness. *Indian J Ophthalmol*. 2012;60:423-427.

4. Hara H, Cooper DK. Xenotransplantation--the future of corneal transplantation? *Cornea*. 2011;30:371-378.

5. Miller TD, Maxwell AJ, Lindquist TD, Requard J, III. Validation of cooling effect of insulated containers for the shipment of corneal tissue and recommendations for transport. *Cornea*. 2013;32:63-69.

6. Kim MJ, Bakhtiari P, Aldave AJ. The international use of the Boston type I keratoprosthesis. *Int Ophthalmol Clin*. 2013;53:79-89.

7. Tay AB, Tan DT, Lye KW, Theng J, Parthasarathy A, Por YM. Osteo-odonto-keratoprosthesis surgery: a combined ocular-oral procedure for ocular blindness. *Int J Oral Maxillofac Surg*. 2007;36:807-813.

8. Cruzat A, Tauber A, Shukla A, Paschalis EI, Pineda R, Dohlman CH. Low-Cost and Readily Available Tissue Carriers for the Boston Keratoprosthesis: A Review of Possibilities. *J Ophthalmol*. 2013;2013:686587.

9. Myung D, Duhamel PE, Cochran JR, Noolandi J, Ta CN, Frank CW. Development of hydrogel-based keratoprostheses: a materials perspective. *Biotechnol Prog*. 2008;24:735-741.

10. Fagerholm P, Lagali NS, Merrett K, et al. A biosynthetic alternative to human donor tissue for inducing corneal regeneration: 24-month follow-up of a phase 1 clinical study. *Sci Transl Med*. 2010;2:46ra61.

11. Ma A, Zhao B, Bentley AJ, et al. Corneal epithelialisation on surface-modified hydrogel implants: artificial cornea. *J Mater Sci Mater Med*. 2011;22:663-670.

12. Fu Y, Fan X, Chen P, Shao C, Lu W. Reconstruction of a tissue-engineered cornea with porcine corneal acellular matrix as the scaffold. *Cells Tissues Organs*. 2010;191:193-202.

13. Lynch AP, Ahearne M. Strategies for developing decellularized corneal scaffolds. *Exp Eye Res*. 2013;108:42-47.

14. Shah A, Brugnano J, Sun S, Vase A, Orwin E. The development of a tissue-engineered cornea: biomaterials and culture methods. *Pediatr Res*. 2008;63:535-544.

15. Griffith M, Polisetti N, Kuffova L, et al. Regenerative approaches as alternatives to donor allografting for restoration of corneal function. *Ocul Surf*. 2012;10:170-183.

16. Fagerholm P, Lagali NS, Ong JA, et al. Stable corneal regeneration four years after implantation of a cell-free recombinant human collagen scaffold. *Biomaterials*. 2014;35:2420-2427.

17. Myung D, Koh W, Bakri A, et al. Design and fabrication of an artificial cornea based on a photolithographically patterned hydrogel construct. *Biomed Microdevices*. 2007;9:911-922.

18. Lodsih H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, Darnell J. Collagen: The Fibrous Proteins of the Matrix. In: *Molecular Cell Biology*. 4th ed. New York: Freeman, W.H.; 2000.

19. Lee KY, Mooney DJ. Hydrogels for tissue engineering. *Chem Rev*. 2001;101:1869-1879.

20. Ahn JI, Kuffova L, Merrett K, et al. Crosslinked collagen hydrogels as corneal implants: effects of sterically bulky vs. non-bulky carbodiimides as crosslinkers. *Acta Biomater*. 2013;9:7796-7805.

21. Song E, Yeon KS, Chun T, Byun HJ, Lee YM. Collagen scaffolds derived from a marine source and their biocompatibility. *Biomaterials*. 2006;27:2951-2961.

22. Krishnan S, Sekar S, Katheem MF, Krishnakumar S, Sastry TP. Fish scale collagen--a novel material for corneal tissue engineering. *Artif Organs*. 2012;36:829-835.

23. Lin CC, Ritch R, Lin SM, et al. A new fish scale-derived scaffold for corneal regeneration. *Eur Cell Mater*. 2010;19:50-57.

24. Chen MH, Li YH, Chang Y, et al. Co-induction of hepatic IGF-I and progranulin mRNA by growth hormone in tilapia, Oreochromis mossambiccus. *Gen Comp Endocrinol*. 2007;150:212-218.

25. Okuda M, Ogawa N, Takeguchi M, et al. Minerals and aligned collagen fibrils in tilapia fish scales: structural analysis using dark-field and energy-filtered transmission electron microscopy and electron tomography. *Microsc Microanal*. 2011;17:788-798.

26. van Essen TH, Lin CC, Hussain AK, et al. A fish scale-derived collagen matrix as artificial cornea in rats: properties and potential. *Invest Ophthalmol Vis Sci*. 2013.

27. Liu J, Song G, Wang Z, et al. Establishment of a corneal epithelial cell line spontaneously derived from human limbal cells. *Exp Eye Res*. 2007;84:599-609.

28. He M, Wang W, Zhang X, Huang W. Ologen implant versus mitomycin C for trabeculectomy: a systematic review and meta-analysis. *PLoS One*. 2014;9:e85782.

29. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods*. 2012;9:671-675.

30. Maurice DM. Nutritional aspects of corneal grafts and prostheses. In: Rycroft PV (ed), *Corneo-Plastic Surgery: Proceedings of the Second International Corneo-Plastic Conference held at the Royal College of Surgeons of England, London, July 1967* London: Pergamon Press; 1969:197-207.

31. Hjortdal JO. Extensibility of the normo-hydrated human cornea. *Acta Ophthalmol Scand*. 1995;73:12-17.

32. Tian X, Liu Z, Niu B, et al. E-cadherin/beta-catenin complex and the epithelial barrier. *J Biomed Biotechnol*. 2011;2011:567305.

33. Garrod D, Chidgey M. Desmosome structure, composition and function. *Biochim Biophys Acta*. 2008;1778:572-587.

34. Stepp MA, Spurr-Michaud S, Tisdale A, Elwell J, Gipson IK. Alpha 6 beta 4 integrin heterodimer is a component of hemidesmosomes. *Proc Natl Acad Sci U S A*. 1990;87:8970-8974.

35. de Pereda JM, Ortega E, Alonso-Garcia N, Gomez-Hernandez M, Sonnenberg A. Advances and perspectives of the architecture of hemidesmosomes: lessons from structural biology. *Cell Adh Migr*. 2009;3:361-364.

36. Nishiuchi R, Takagi J, Hayashi M, et al. Ligand-binding specificities of laminin-binding integrins: a comprehensive survey of laminin-integrin interactions using recombinant alpha3beta1, alpha6beta1, alpha7beta1 and alpha6beta4 integrins. *Matrix Biol*. 2006;25:189-197.

37. Masur SK, Cheung JK, Antohi S. Identification of integrins in cultured corneal fibroblasts and in isolated keratocytes. *Invest Ophthalmol Vis Sci*. 1993;34:2690-2698.

38. Robert MC, Biernacki K, Harissi-Dagher M. Boston keratoprosthesis type 1 surgery: use of frozen versus fresh corneal donor carriers. *Cornea*. 2012;31:339-345.

39. Tan A, Tan DT, Tan XW, Mehta JS. Osteo-odonto keratoprosthesis: systematic review of surgical outcomes and complication rates. *Ocul Surf*. 2012;10:15-25.

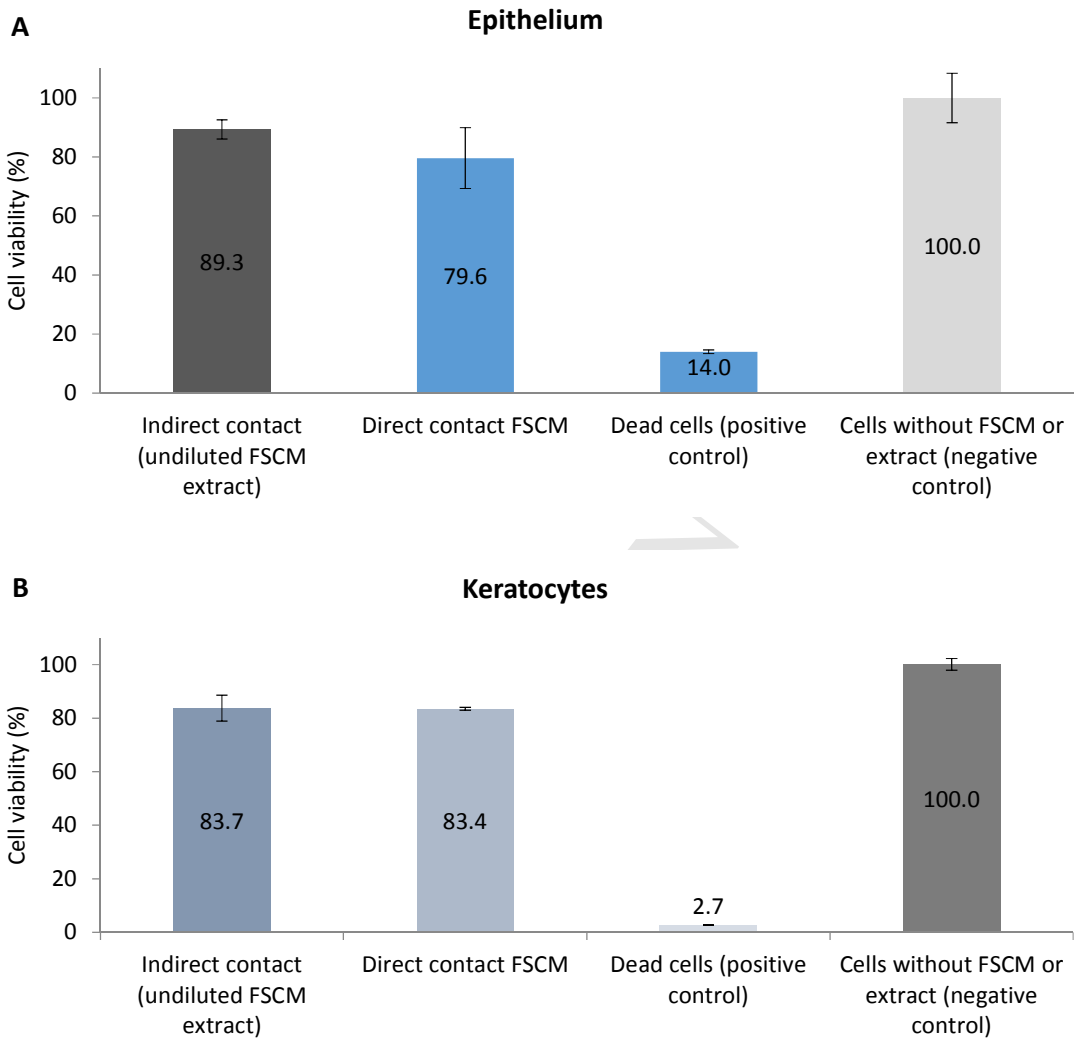
40. Schrage N, Cordes AK, Storsberg J, et al. [In vitro and in vivo investigations of a modified textile keratoprosthesis. ACTO TexKPRO]. *Ophthalmologe*. 2014;111:247-253.

41. Luo ZL, Vanchinathan V, Dalal R, et al. Biocompatibility of poly(ethylene glycol) and poly(acrylic acid) interpenetrating network hydrogel by intrastromal implantation in rabbit cornea. *J Biomed Mater Res A*. 2015.

42. Hartmann L, Watanabe K, Zheng LL, et al. Toward the development of an artificial cornea: improved stability of interpenetrating polymer networks. *J Biomed Mater Res B Appl Biomater*. 2011;98:8-17.

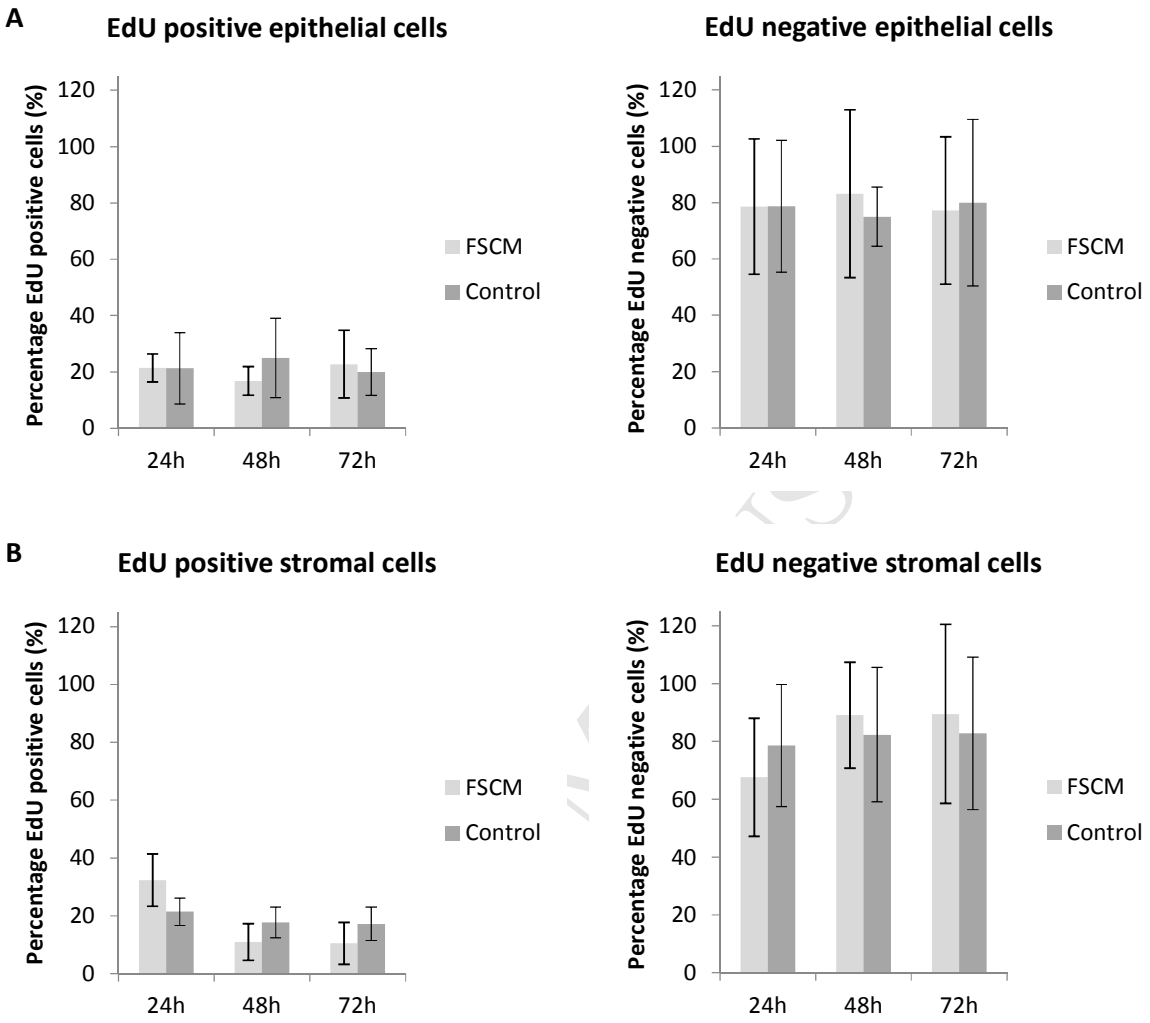
Figures

Figure 1



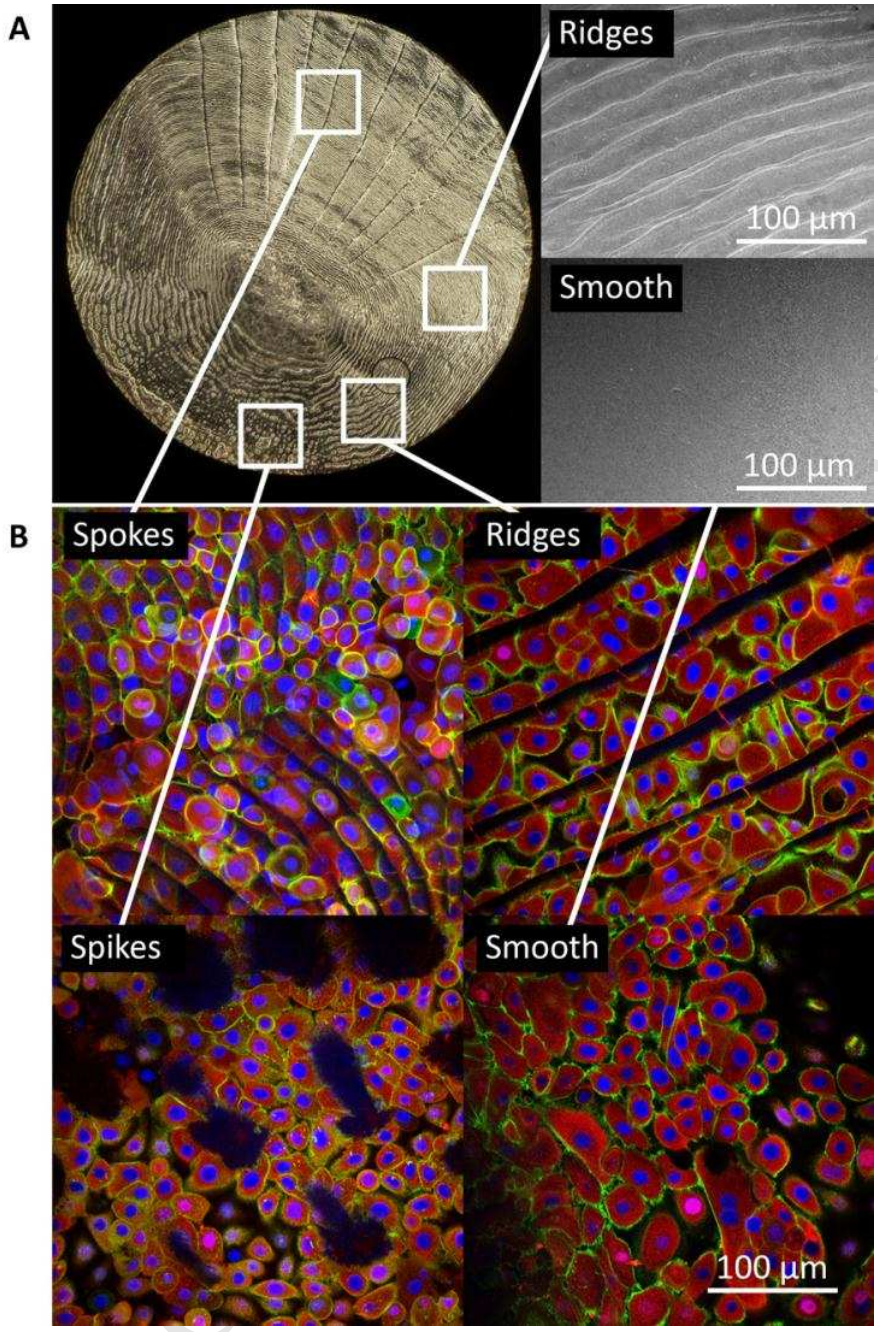
(one column) Figure 1. Effect of fish scale-derived collagen matrix (FSCM) on cellular viability. Primary human corneal epithelial cells (A) or primary human stromal cells (B) were cultured in indirect and direct contact with the fish scale-derived collagen matrix (FSCM), and the cytotoxicity was tested using a viability assay (MTT test). As a positive control for minimal MTT staining (toxicity) we used cells killed with SDS.

Figure 2



(one column) Figure 2. Cell proliferation of human corneal epithelial and stromal cells cultured on FSCM or plastic. Primary human corneal epithelial cells (A) or primary human stromal cells (B), were cultured on the fish scale-derived collagen matrix (FSCM) or on plastic control discs and proliferation was determined in a three-day proliferation assay, in which actively dividing cells were stained with 1% 5-ethynyl-2'-deoxyuridine (EdU). There were no significant differences between the percentage EdU positive and EdU negative epithelial and stromal cells grown on the FSCM and on the control discs (Mann-Whitney U test).

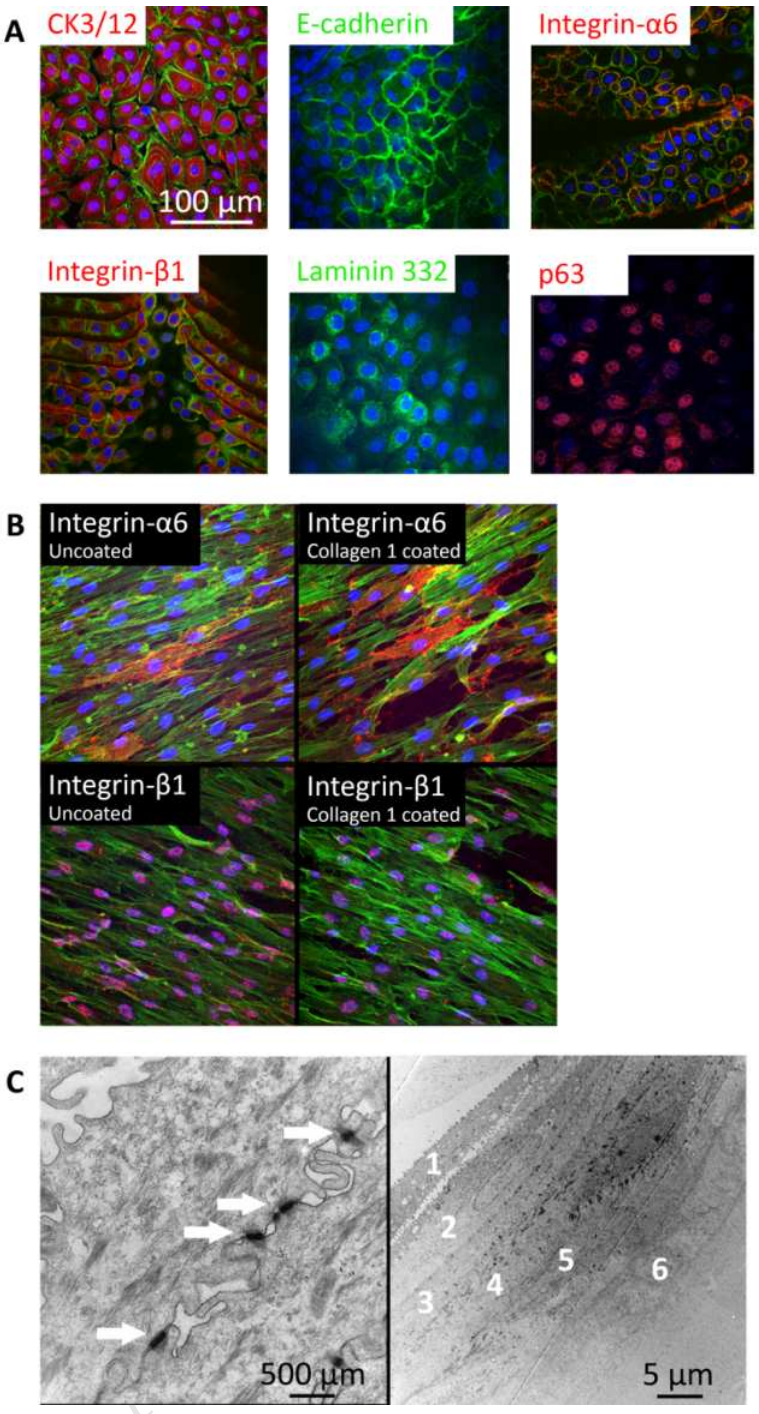
Figure 3



(one column) Figure 3. In vitro human corneal epithelial cell growth on the FSCM The anterior surface of the FSCM has three distinguishable patterns: spokes, ridges, and spikes, as clearly seen with a phase-contrast microscope, while the posterior surface is smooth, as demonstrated by a SEM (A). The primary human corneal epithelial cells grew equally on each surface (B).

Blue: DAPI, staining cell nuclei; Green: Alexa Fluor 488 Phalloidin, staining F-actin; Red: CK3/12, staining differentiated corneal epithelium.

Figure 4



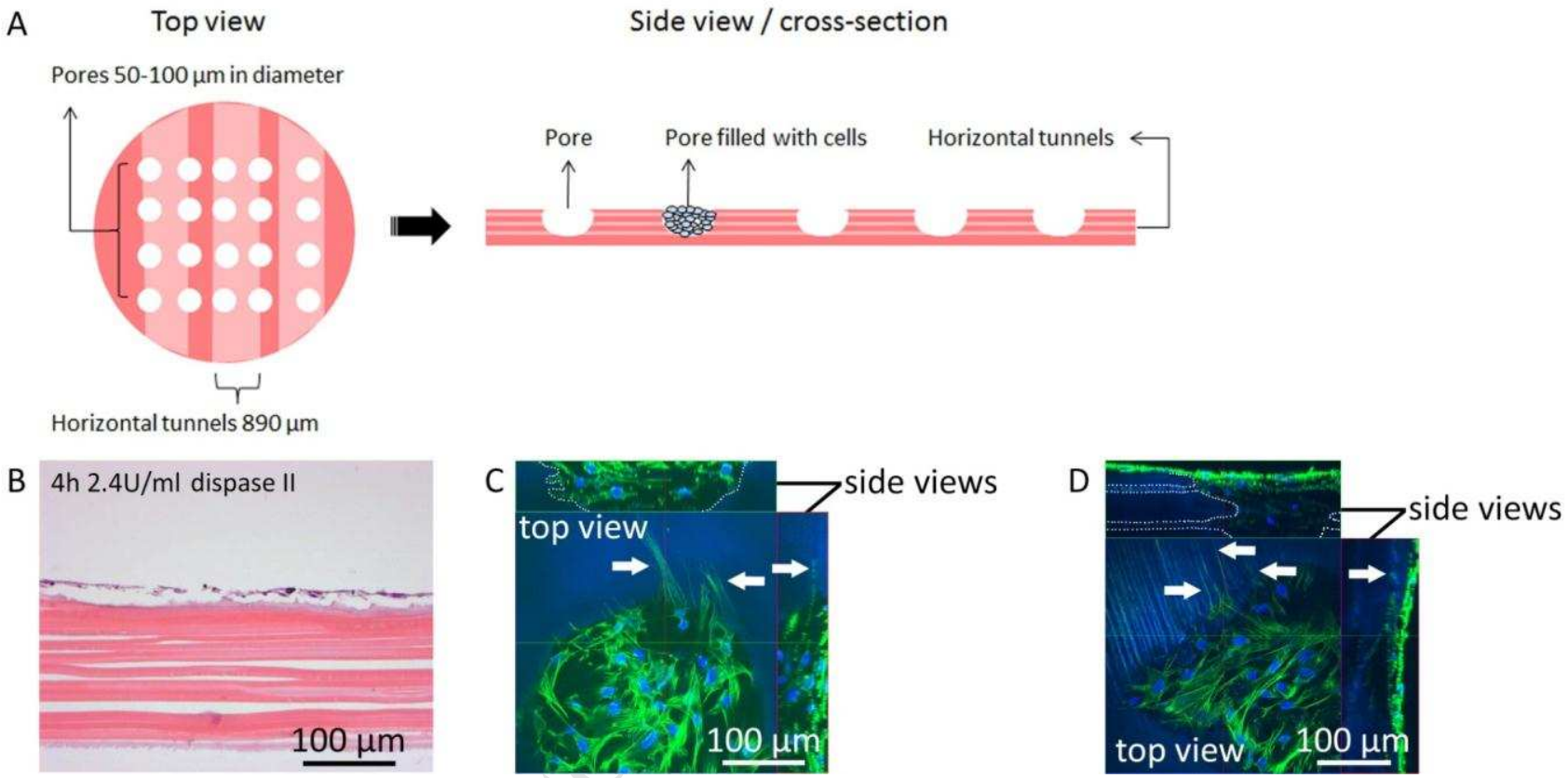
(one column) Figure 4. In vitro characteristics of primary human corneal epithelial cells grown on FSCM.

A. Primary human corneal epithelial cells cultured on the FSCM stained positive for CK3/12 (red) (indicating differentiated epithelium), for the cell-cell adhesion marker E-cadherin (green), for the

cell-matrix/cell-cell adhesion markers integrin-α6 (red), integrin-β4 (red), and basement membrane marker laminin 332 (green), and most cells stained positive for the stem cell marker p63 (red) (A; blue = cell nuclei; green = F-actin, unless otherwise stated).

B. Primary human corneal stromal cells, the keratocytes, displayed the adhesion molecules integrin-α6 and integrin-β1, whether the FSCM was coated with collagen type I or not (B; blue = cell nuclei, green = F-actin, red = integrins). C. Desmosomes were present (arrows) between the epithelial cells cultured on the FSCM, and stratification occurred (C; numbers indicate cell layers).

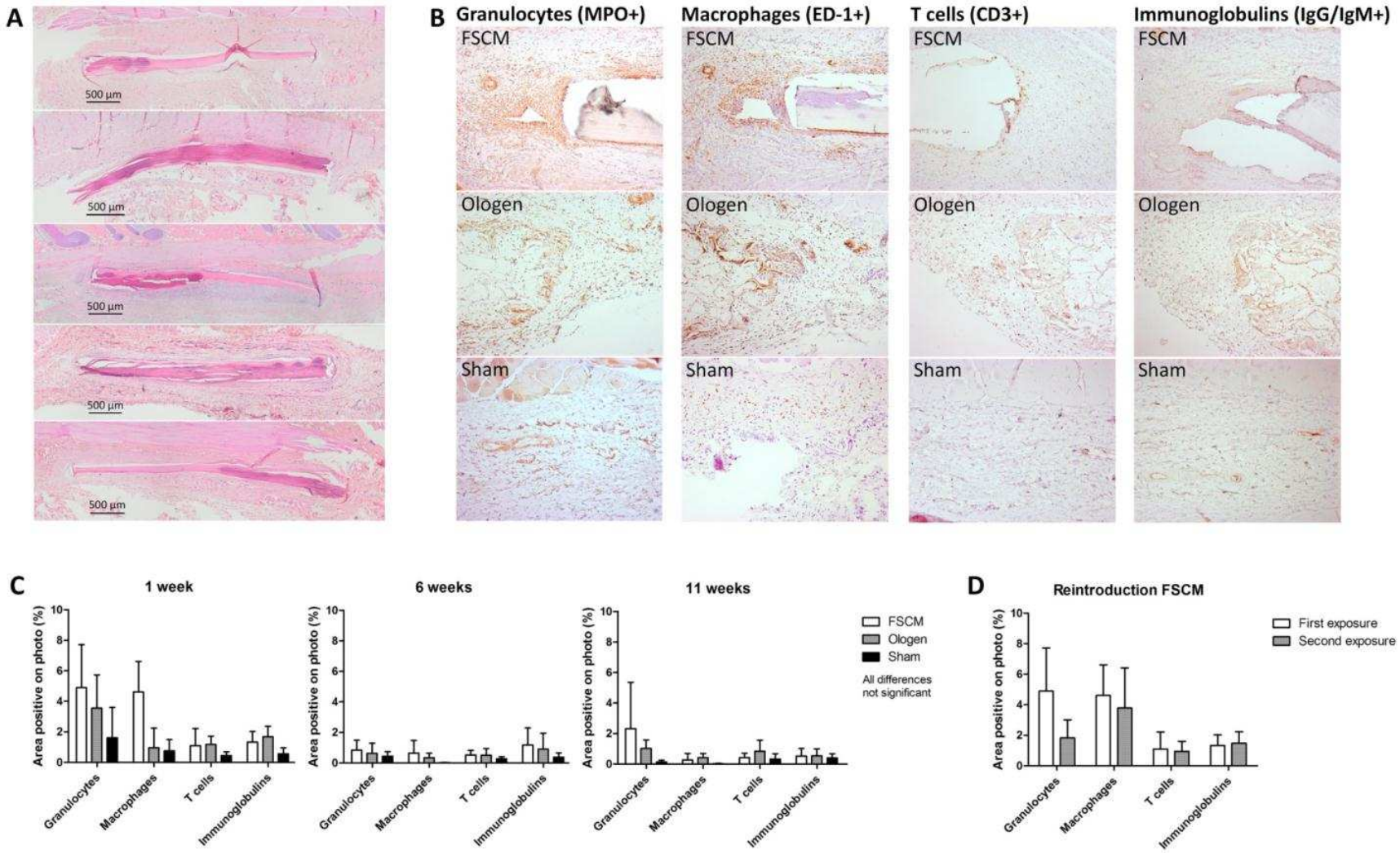
Figure 5



(two column) Figure 5. Modification of the FSCM by generating pores to increase cell ingrowth. The FSCM can be manipulated with the femto-second laser to create pores and horizontal tunnels in the FSCM (A), as well as with dispase II to create a small space between the naturally present horizontal

collagen layers at the outer edge of the FSCM (B). Primary human corneal stromal cells are shown to interact with these alterations by filling up the small spaces (see arrows) thus created by dispase II in FSCMs with laser made pores (C), and in FSCMs in which horizontal tunnels were created subsequently (D). The white dotted lines indicate the borders of the pores (C) and of the pores and tunnels (D) created in the FSCM, and the side views represent digital cross sections.

Figure 6


A
500 µm
500 µm
500 µm
500 µm
500 µm
B
Granulocytes (MPO+)
Macrophages (ED-1+)
T cells (CD3+)
Immunoglobulins (IgG/IgM+)
FSCM
Ologen
Sham
C
1 week
6 weeks
11 weeks
Area positive on photo (%)
Granulocytes
Macrophages
T cells
Immunoglobulins
FSCM
Ologen
Sham
All differences not significant
D
Reintroduction FSCM
First exposure
Second exposure

(two column) Figure 6. FSCM or control Ologen® Collagen Matrices were implanted subcutaneously and tissue were studied for different infiltrating cell types at 1, 6 or 11 weeks after implantation.

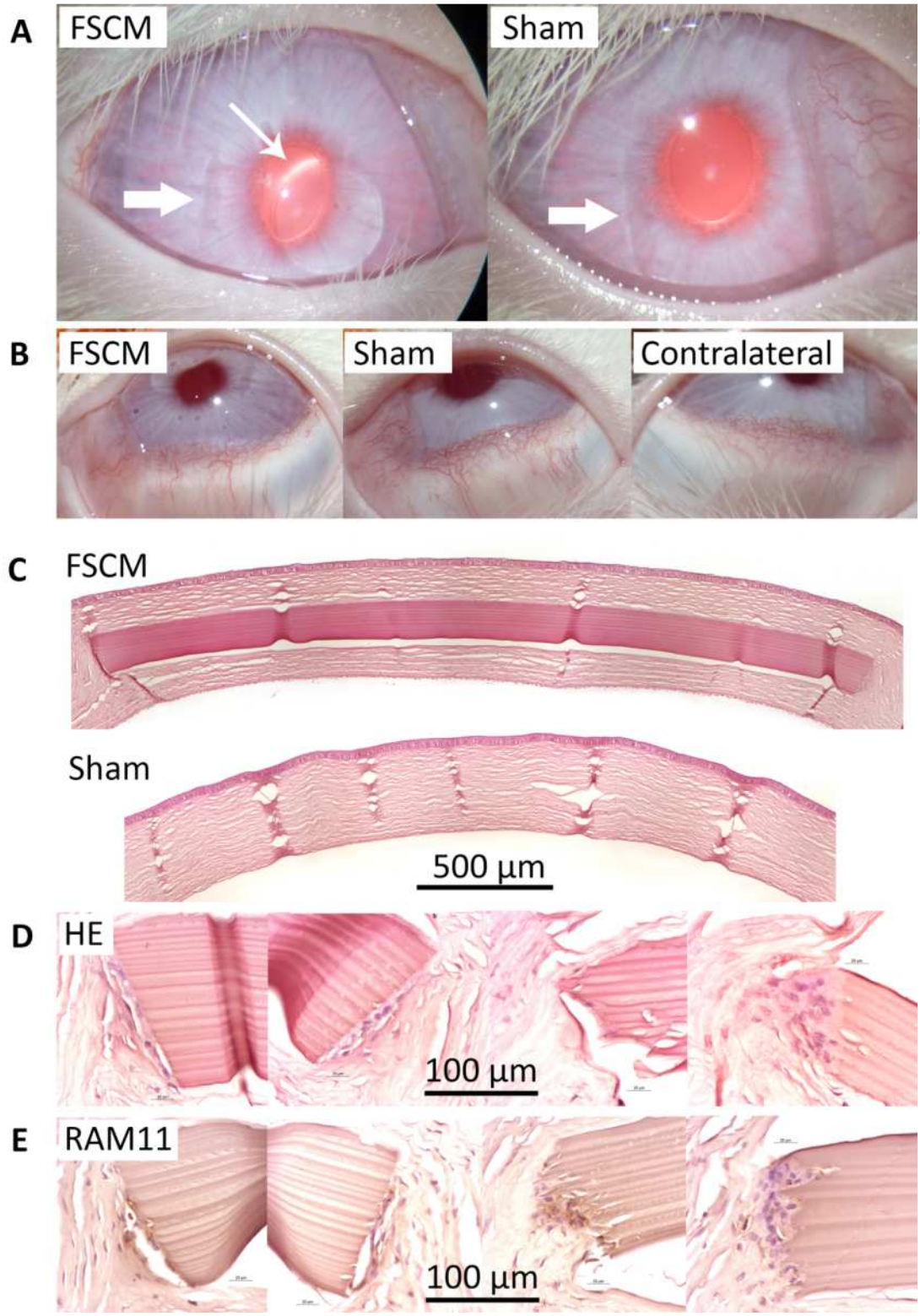
A. Overview of the subcutaneous implanted FSCM and the surrounding tissue at 11 weeks on HE staining.

B. Examples of the immunostaining pictures at one week follow up for granulocytes, macrophages, T-cells, and immunoglobulins for the FSCM, the Ologen® implant and following sham surgery.

C. Staining areas were imaged by microscope (Zeiss Axioskop 40) and calculated with ImageJ. The graphs show the percentage of immunopositive staining areas for each marker, and demonstrate that all average values are below 5% and that they further diminish over time.

D. After reimplantation, no sensitization has occurred as the immune response to the second exposure is similar to the first exposure. The tissue was tested 1 week after reimplantatation.

Figure 7



(one column) Figure 7. In vivo tolerance to FSCMs in rabbit corneas.

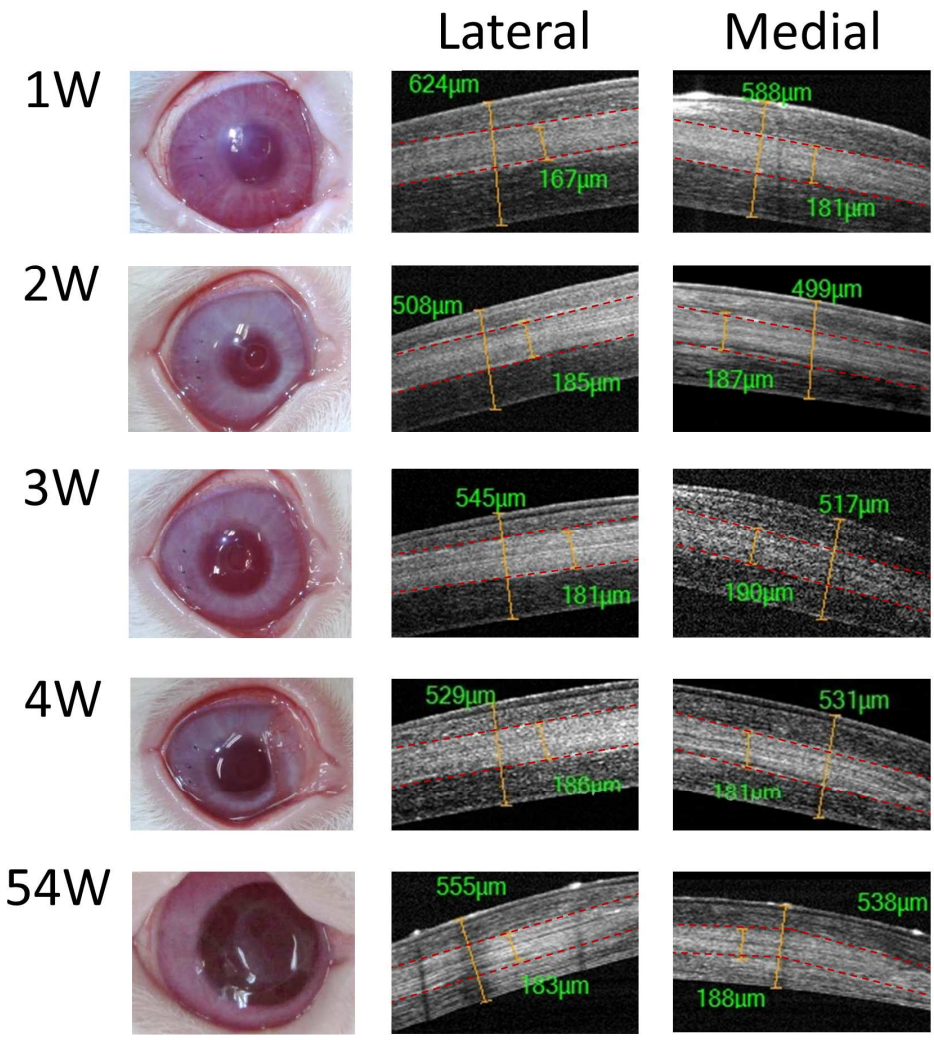
A. Intrastromally implanted FSCMs (small arrow) in rabbits stayed clear during follow-up, and do not differ from the image of the rabbits that received sham surgery (big arrows indicate site of incision).

B. Neovascularization was physiological and was equal in eyes with an FSCM to eyes after sham surgery or contralateral eyes.

C. The overlaying epithelium appeared viable on the HE-staining, and a few infiltrating cells, depending on the location at the FSCM's edge (D), were present.

E. Macrophages (RAM11 staining) were seen in the scarce infiltrate (dark brown is immunopositive).

Figure 8



(one column) Figure 8. Long-term follow up of intrastromal FSCM's

The intrastromally placed FSCM remained stable in situ during a total of 54 weeks (54W) without eliciting an immune response. The first column shows the rabbit eye in vivo, the second column shows a cross section made by OCT of the lateral area of the FSCM and the third column shows a cross section of the medial area of the FSCM. The thickness of the FSCM was stable and showed no signs of degradation. At 54 week some scar-tissue was visible.