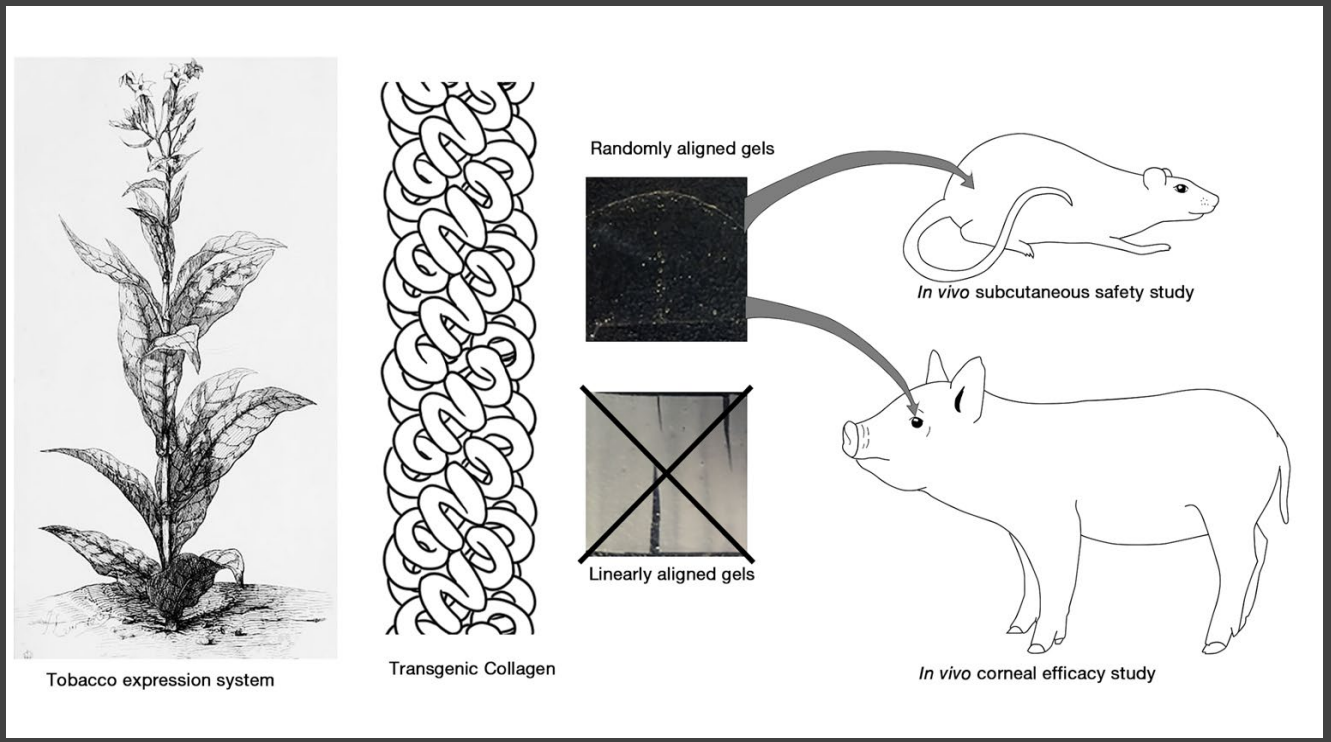
Plant Source Recombinant Human Collagen Type I for Corneal Regeneration

Elle Simpson-Edin, PhD

Includes work by:
Michel Haagdorens1,2, Elle Simpson-Edin3,4, Per Fagerholm6, Marc Groleau4, Zvi Shtein5, Artūras Ulčinas7, Amit Yaari5, Ayan Samanta8, Vytautas Cepla7,10, Aneta Liszka6, Marie-José Tassignon1,2, Fiona Simpson3,4, Oded Shoseyov5,11, Ramūnas Valiokas7, Isabel Pintelon9, Monika Kozak Ljunggren5, May Griffith3,4

1 Department of Ophthalmology, Visual Optics and Visual Rehabilitation, University of Antwerp, Antwerp, Belgium.
2 Department of Ophthalmology, Antwerp University Hospital, Antwerp, Belgium
3Department of Ophthalmology and Institute of Biomedical Engineering, University of Montreal, Montreal, Quebec, Canada
4Maisonneuve-Rosemont Hospital Research Centre and CHUM Research Centre, Montreal, Quebec, Canada
5The Robert H. Smith Faculty of Agriculture, Food and Environment, and the Center for Nanoscience and Nanotechnology, The Hebrew University of Jerusalem, Jerusalem, Israel
6Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden
7Dept. of Nanoengineering, Center for Physical Sciences and Technology, Vilnius, Lithuania
8 Department of Chemistry – Ångström Laboratory, Uppsala, Sweden
9Laboratory of Cell Biology and Histology, Antwerp University, Antwerp, Belgium
10Ferentis UAB, Vilnius, Lithuania
11 CollPlant Ltd., Ness-Ziona, Israel



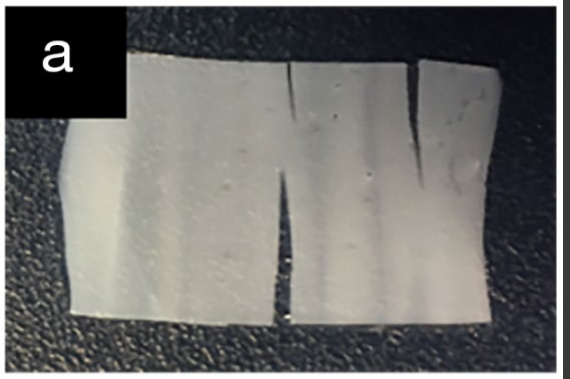
A severe shortage of human donor corneas for transplantation that has led scientists to develop synthetic alternatives. Here, recombinant human collagen type I (RHCI) made in tobacco plants through genetic engineering was tested for use in making corneal implants.

Transgenic pipeline:

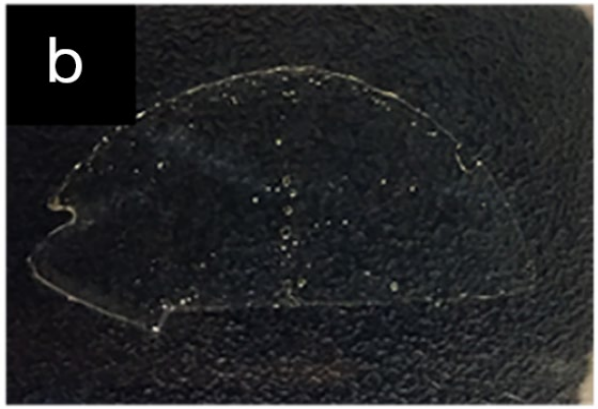
- Allow circumventing risks of zootropic disease
- Simplifies industrial development through non-reliance on sources that are sensitive to disease outbreaks, like secondary products from the meat industry.

Recombinant Collagen Type I (RHCI) hydrogels with random fibrillogenesis were fabricated (rRHCI).
Fibrillogenesis was initiated by addition of sodium phosphate buffer that was adjusted to pH 7.5 with NaOH. EDC and NHS were added to crosslink the fibrils. The RHCI suspension was dispensed into a rectangular Teflon mould (4.5 x 4.5 cm) for *in vitro* characterization, or into curved corneal-shaped and sized as implants. The collagen was then air-dried under a sterile hood and immersed in 100 % ethanol to promote detachment from the mould. The gels were stored in 100 % ethanol until further use. Gels were rehydrated by five 2-hour soaks in phosphate-buffered saline (PBS).

Aligned RHCI



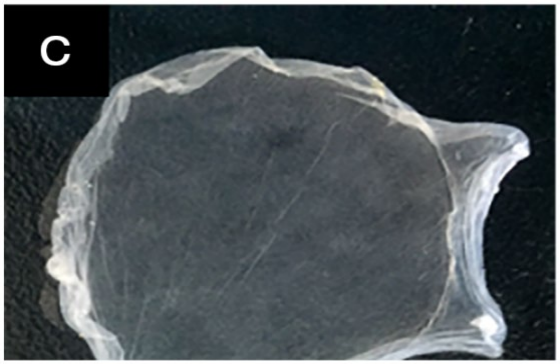
Random RHCI

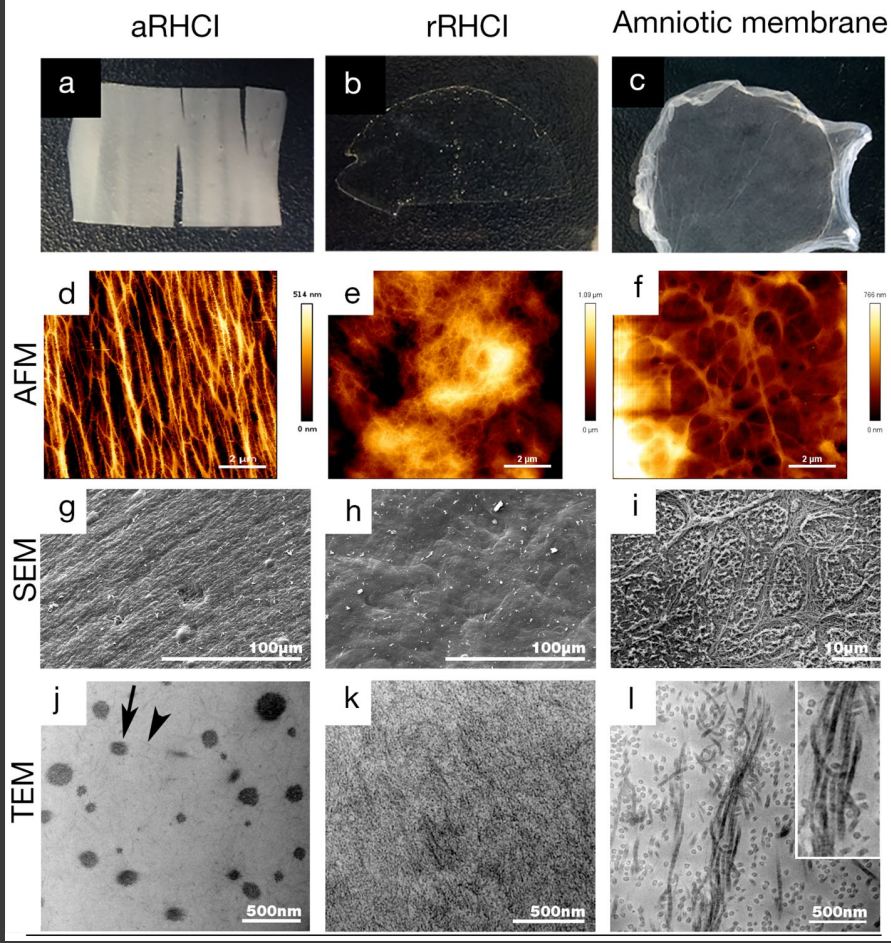


For production of aligned RHCI membranes (aRHCI), shear force was applied to the collagen solution prior to fibrillogenesis and chemical crosslinking. Shear force was created by using a motorized film applicator, Elcometer 4340 (Elcometer Ltd, Manchester, UK) and an Elcometer 3570 'Doctor Blade' that served as the orthogonal force. After ethanol dilution, the acidic RHCI was cast onto a glass plate that was mounted in the Elcometer. The glass plate had previously been surface-coated with a hydrophobic siloxane solution. After collagen casting, shear force was applied at a constant speed of 0,02 m/s and a thickness of 50 µm. After the RHCI had spread, the glass plate was immediately lifted from the Elcometer and immersed in a bath of fibrillogenesis buffer. Membranes were then crosslinked for 24 hours in the same manner as the random gels. Hydrogels were kept in 100% ethanol until further use.

Human amniotic membrane (HAM) was used as a benchmark. HAM was washed in an antibiotic cocktail and then flattened onto sterilized nitrocellulose filter paper, epithelial side up. The paper-supported HAM was then cut into 5cm x 5cm pieces and cryopreserved at -80°C in 50% Dulbecco's Modified Eagle Medium (DMEM), 50% glycerol. Prior to use, HAM was thawed, epithelial cells were removed, and the cell-free membrane was washed. For use, HAM is oriented spongy layer up, after which excessive water is mechanically expelled using cell scrapers. For stabilization, HAM was mounted 'spongy layer' down in a CellCrown or interlockable ring.

Amniotic membrane





Properties	aRHCI	rRHCI	HAM	Human Cornea[1]
Water content (%)	92.21 ± 0.007	89.21 ± 0.003	87.68 ± 0.018	78
Transmission (%)	37.1	91.5	65.9	87
Tensile strength (MPa)	*	2.67±0.8	7.6±2.4	3.81 ± 0.4
Elongation at break (%)	*	28±6	68±10	**
Young's Modulus (MPa)	*	52.79±13.1	23.14±4.34	3-13

1: Fagerholm P, Lagali NS, Ong JA, Merrett K, Jackson WB, Polarek JW et al. Biomaterials. 2014;35(8):2420-7.

Structural properties of RHCI hydrogels compared to human amniotic membrane (HAM).

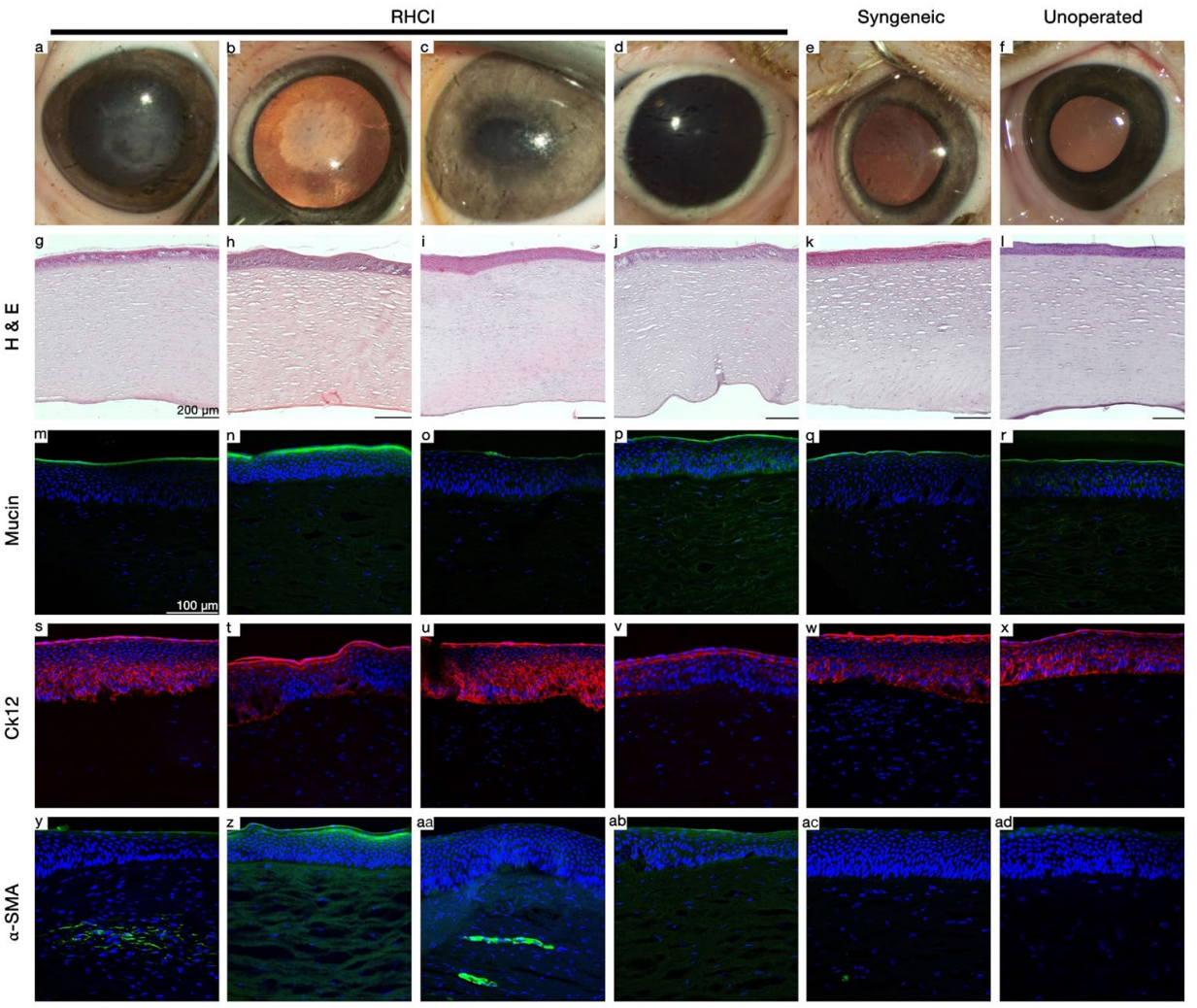
(a) Aligned hydrogels are semi-translucent and have the tendency of splitting unidirectionally.

(b) Random collagen hydrogels are optically transparent.

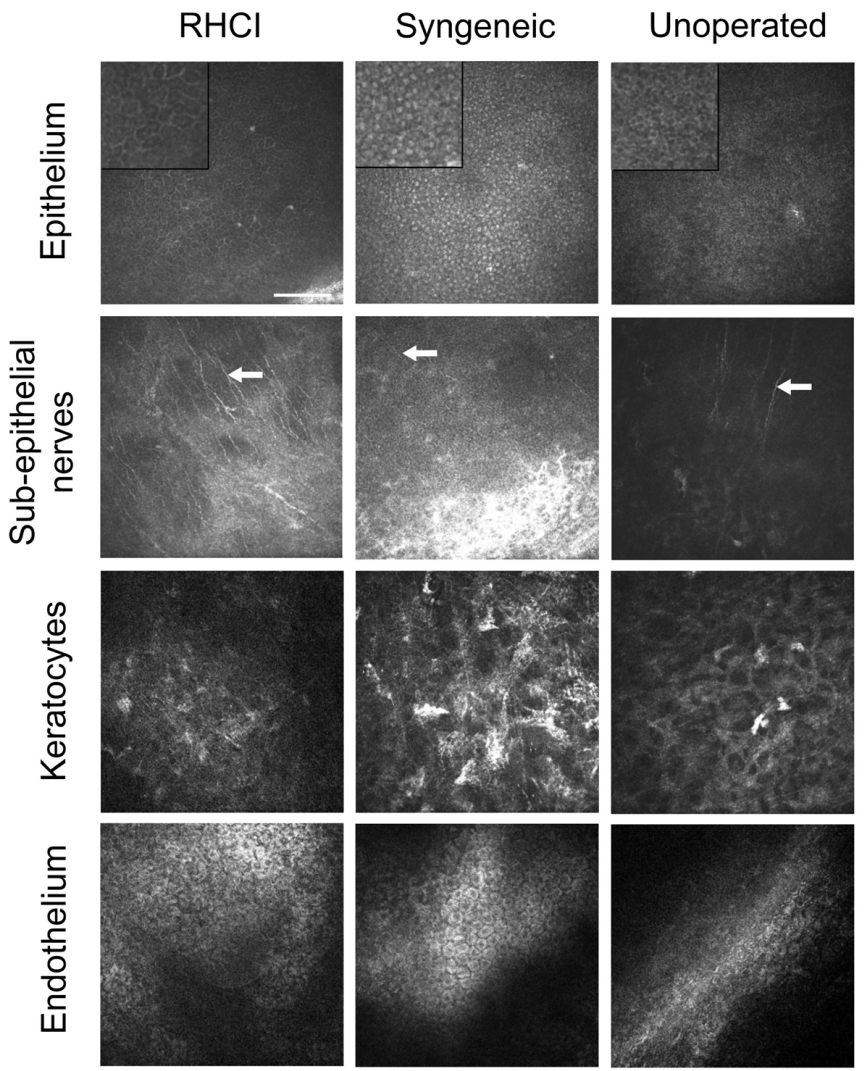
(c) Control HAM serves as reference.

AFM (**d-f**) and SEM (**g-i**) of hydrogels, showing that in random hydrogels, very fine collagen fibrils were present, resulting in a smooth surface as revealed by SEM. Cross-sectional TEM of the hydrogels with aligned fibers (**j**) showed thick RHCI fibers (arrow) interspersed with thin collagen fibrils (arrowhead). The collagen fibers show unidirectional alignment, whereas the interpenetrating fibrils are randomly dispersed (**k**). Fibers were of varying thickness, 75-200 nm diameter. In random hydrogels, only fine collagen fibrils are seen, although some clumping was seen in the AFM and TEM images. (**l**) Collagen fibers were randomly oriented in HAM, with fibers showing distinct collagen banding (inset). The inset table shows the optical and mechanical characteristics of RHCI hydrogels and human amniotic membrane (HAM). * The stress test was not performed on the aligned RHCI hydrogels. **not performed.

Columns 1-4 shows each of the individual animals in the RHCI group. Column 5 shows representative images from the syngeneic graft. Column 6 shows representative images from unoperated controls. (a-f) Eyes of all RHCI grafted pigs and controls, showing haze in a and b. (g-l) Haematoxylin and eosin stained sections show morphologies of regenerated neo-corneas are comparable to those of the controls. Mucin staining (m-r) shows that the animals in the RHCI group have intact tear films. Cytokeratin 12(Ck12) staining (s-x) shows differentiated corneal epithelial cells in all samples. (y-ad) α-smooth muscle actin (α-SMA), a myofibroblast marker shows the presence of activated fibroblasts in RHCI corneas, particularly in the ones with haze.



In vivo confocal microscope images of RHCI and syngeneic grafted corneas, compared to unoperated healthy corneas. Scale bars, 100 µm. Insets, 100 x 100 µm. The insets show epithelial cell morphology in detail. Sub-epithelial nerves (arrows) in regenerated RHCI implanted corneas form parallel bundles like those of unoperated corneas, while those of syngeneic grafts were disorganized. Stromal keratocytes were present in all three groups. The endothelial layers of operated corneas retained a healthy morphology.



Funding

This research was funded with research grants from The Research Foundation Flanders (#FWO - 11ZB315N) to MH; EuroNanoMed II (G0D5615N to MJT) for the REGENERATE project in partnership with the Swedish Research Council (529-2014-7490 to MKL) and the Research Council of Lithuania (EuroNanoMed2-01/2015 to RV), and the European Cooperation in Science and Technology (EU-COST BM1302). MG acknowledges salary and research support from her Canada Research Chair Tier 1 in Biomaterials and Stem Cell in Ophthalmology and a Caroline Durand Foundation Research Chair in Cellular Therapy in the Eye, as well as a Honorary Professorship at the School of Optometry and Vision Science, Cardiff University. FS is supported by an NSERC Canada doctoral studentship.