FP6

PRIORITY 3 - Nanotechnologies and nanosciences, knowledge-based multifunctional materials and new production processes and devices (NMP)

NMP2-CT-2003-504017

CORNEA ENGINEERING
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THREE-DIMENSIONAL RECONSTRUCTION OF HUMAN CORNEAS BY TISSUE ENGINEERING

Publishable final activity report

Period covered: from 01/01/2004 to 31/12/2007 Date of preparation: 15/02/2007

Start date of project: 01/01/2004 Duration: 48 months

Project coordinator name : D.J.S. Hulmes
Project coordinator organisation name : CNRS

Project funded by the European Commission under contract n°NMP2-CT-504017

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1. Introduction

Approximately 10 million people worldwide are blind as a result of corneal diseases. At the moment, the only generally available treatment option is to replace corneas using donor tissue. In Europe, approximately 25,000 such operations are carried out each year. But there is an increasing risk of disease transmission from donor tissue and furthermore the growing use of laser corrective surgery renders corneas unsuitable for grafting. These factors, together with the limited applications of synthetic polymer based artificial corneas (keratoprostheses), point to the urgent need to develop tissue engineered corneas for clinical applications. In addition, following recent European Directives banning the use of animals for toxicity testing, there is an urgent requirement to develop in vitro alternatives to the widely used Draize eye test.

The cornea is a specialised connective tissue whose structure is adapted to its principal functions of focussing light and resisting intraocular pressure (Fig. 1). This is achieved by the multi-layer organisation of the stroma (about 500 µm thick) which consists of about 200 layers consisting of cells (keratocytes) surrounded by a dense extracellular matrix (ECM). Within each layer, narrow diameter collagen fibrils are aligned in parallel, separated by proteoglycans and other ECM components. Between successive layers, there is an abrupt change in orientation giving rise to a plywood-like structure. The stroma is covered on the external surface by a stratified epithelium, made of keratinocytes, and on the internal surface by a layer of endothelial cells.

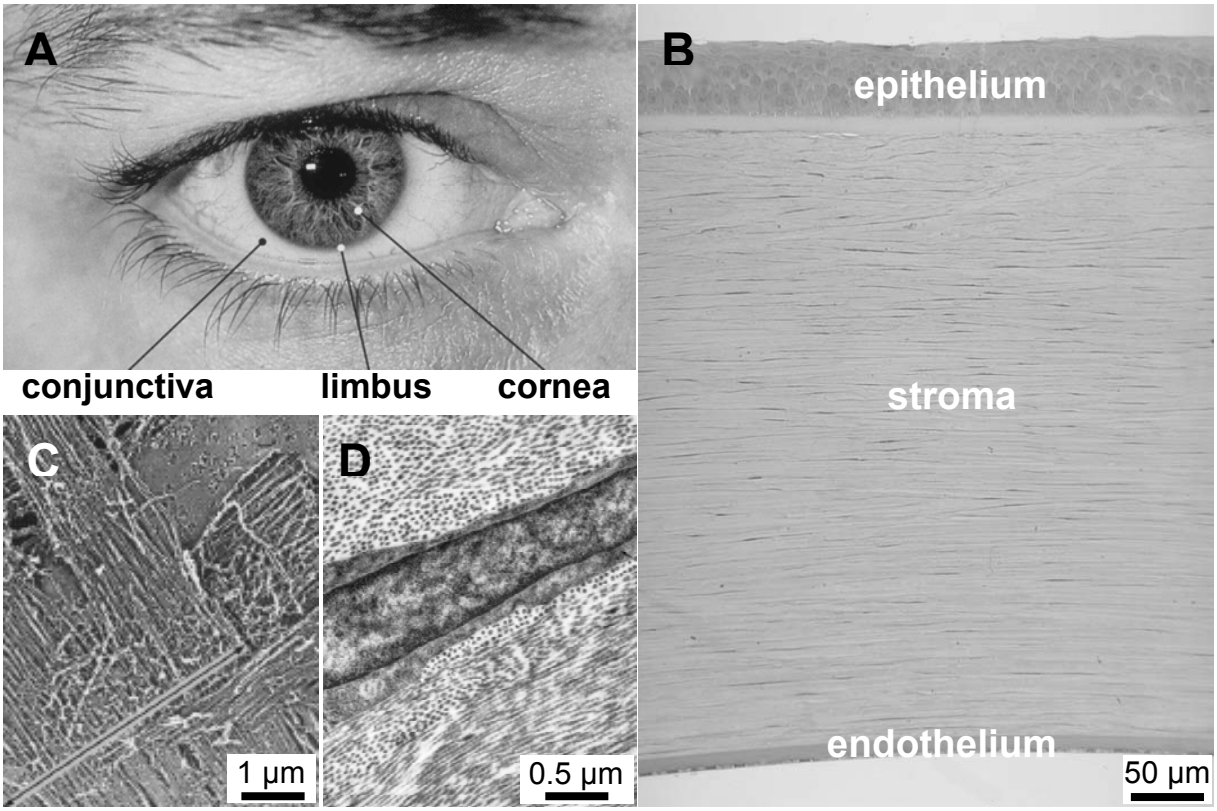

Figure 1. Structure of the cornea showing (A) the surrounding conjunctiva and limbus, (B) the epithelium, stroma and endothelium and (C,D) the lamellar arrangement of collagen fibrils in the stroma by (C) scanning and (D) transmission electron microscopy, the latter also showing part of a stromal keratocyte.

Tissue engineering is a multidisciplinary subject that aims to restore tissue function either by engineering tissues in the laboratory using different cell types supported on three-dimensional scaffolds. Such an approach has already led to clinical applications in the areas of tissue engineered skin, blood vessels, cartilage and bone. A variety of tissue engineered corneas have been described (Germain et al., 1999; Griffith et al., 1999; Reichl et al., 2004), though in none of these has there been any attempt to reconstruct the highly organised structure of the native corneal stroma. As a result, such artificial corneas lack the mechanical strength that required for *in vivo* use. In addition, immortalized cell lines are usually used for the epithelial and endothelial cell layers, making such corneas unsuitable for clinical applications.

The overall aim of this project was to carry out research leading to the three-dimensional reconstruction of human corneas. In order to do so, part of the project was aimed at producing three-dimensional cell scaffolds resembling as close as possible the natural ECM. This required production of recombinant human ECM proteins and their processing enzymes, including functional studies on the roles of these enzymes and associated proteins. In parallel with these studies, another goal was to isolate and characterise the different cell types and identify as far as possible corresponding adult stem cell sources. Part of this work included clinical trials using stem cell-derived epithelial cells. Cell interactions with the ECM were the topic of a further area of research, such interactions being essential for optimal tissue reconstruction. For the tissue engineering, the first aim was to reconstruct a hemi-cornea (epithelium + stroma) as an *in vitro* alternative to animal toxicity testing. Subsequently, a variety of novel scaffolds and hemi-corneas were reconstructed for possible long term clinical applications. Biocompatibility testing in animal models was begun. Finally, full depth corneas were reconstructed *in vitro* using all three cell layers.

2. Extracellular matrix

The ECM of the corneal stroma consists mainly of different types of collagens (types I, V and VI) and proteoglycans. The latter include the keratan sulphate proteoglycans lumican, keratocan and osteoglycin, and the chondroitin/dermatan sulphate proteoglycan decorin. Between the stroma and the epithelium is a thin basement membrane consisting of collagens (types IV, VII, XVII, XVIII), heparan sulphate proteoglycans, nidogens and laminins. Bowman's membrane is a thin acellular region beneath the epithelial basement membrane, while Descemet's membrane (rich in collagen VIII) is at the stroma-endothelium interface.

Fibrillar collagens such as collagens I and V are synthesized in precursor form, procollagens, with large N- and C-terminal propeptides (Birk and Bruckner, 2005; Ricard-Blum et al., 2005). These propeptides are removed by specific extracellular metalloproteases belonging to the ADAMTS (N-terminal processing) and tolloid/BMP1 (mainly C-terminal processing) families (Hopkins et al., 2007). Fibril formation occurs spontaneously following cleavage of the propeptides.

During the project, procedures were devised for the high level expression of human recombinant ADAMTS2 in 293 cells (Colige et al., 2005). We found that the enzyme undergoes activation by furin cleavages in the N-terminal pro-region, as well as further activation by processing of the C-terminal PNP domain. Several different forms of the enzyme were identified both in cell culture and *in vivo*, the most active form lacking both N- and C-terminal domains, but possessing two thrombospondin type repeats required for optimum activity (Fig. 2). In addition to procollagen types I-III, recombinant ADAMTS2 was found to cleave the N-terminal region of homotrimeric procollagen V, at a site C-terminal to that previously shown to be cleaved by BMP1 (Fig. 2). In related work, we showed that naturally occurring mutations in the N-terminal region of the proα1(I) chain of procollagen I,

associated with combined osteogenesis imperfecta/Ehlers Danlos syndrome, lead to defective processing by ADAMTS2 (Cabral et al., 2005). The importance of ADAMTS2 in the regulation of collagen deposition was further demonstrated in a mouse model of liver fibrosis, which was reduced by inactivation of the corresponding gene (Kesteloot et al., 2007). More recently, we showed that ADAMTS2 production by monocytes and macrophages is stimulated by glucocorticoids, pointing to a role for these cells in the resolution of inflammation and wound repair (Hofer et al., 2008).

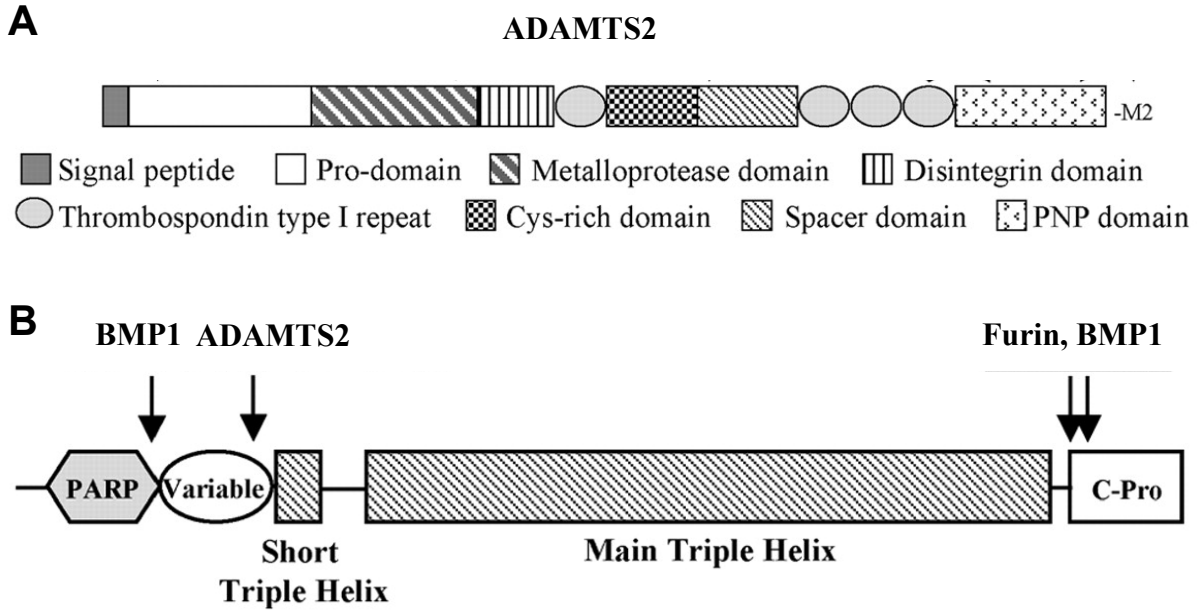

Figure 2. (A) Domain structure of ADAMTS2 and (B) cleavage sites and enzymes involved in proteolytic processing of homotrimeric procollagen V. From Colige et al., 2005.

N-terminal processing of fibrillar procollagens by tolloid/BMP1 proteases is unusual. Normally these enzymes removed the C-propeptide regions from the fibrillar procollagens, but in the case of the minor fibrillar procollagens (types V and XI) they also cleave in the N-terminal regions. In addition, the amino acid sequence cleaved by BMP1 in the N-terminal region of procollagen V, ser-gln, differs from most other BMP1 cleavage sites characterised by an invariant aspartate at the P1' position. Using a co-transfection approach coupled with site-directed mutagenesis, we identified an aspartate residue in the P2' position which is essential for activity, unlike the ser-gln sequence which can be mutated to ala-ala without affecting BMP1 cleavage (Bonod-Bidaud et al., 2007). Collagen V is known to play a major role in corneal structure and function. To investigate the distribution of collagen V in cornea and other tissues during stages of mouse development, we carried out in situ hybridisation studies (Roulet et al., 2007). In addition to its presence throughout the stroma, collagen V expression was found to be relatively high in the region of the limbus (Fig. 1).

The activity of tolloid/BMP1 proteases during cleavage of the procollagen C-propeptides can be enhanced by other ECM proteins known as PCPEs (Hopkins et al., 2007) which themselves are devoid of intrinsic enzymatic activity. In addition to procollagens, tolloid/BMP1 proteases have been shown to cleave several extracellular substrates, including prolysyl oxidases (involved in cross-linking), small leucine rich repeat proteoglycans (biglycan, osteoglycin), basement membrane proteins (perlecan, procollagen VII, laminin 332), growth factors (myostatin, prolactin, growth hormone) and their antagonsists (chordin, latent transforming growth factor binding proteins). To investigate the specificity of PCPE1,

its effect on a range of BMP1 substrates was tested (Moali et al., 2005). We found that only C-terminal processing of fibrillar procollagens was enhanced by PCPE1, thereby identifying a novel class of substrate-specific protease activators. Subsequent molecular modelling and site-directed mutagenesis studies revealed the importance of specific amino acid residues in the CUB1 domain of PCPE1 in enhancing activity (Fig. 3; Blanc et al., 2007).

Collagen interactions with small leucine rich repeat proteoglycans (SLRPs) have been shown to be essential for the maintenance of corneal transparency (Chakravarti et al., 1998). To identify the recognition sites involved, we carried out interaction studies using recombinant fragments and site-directed mutants of decorin (Kalamajski et al., 2007) and fibromodulin (Kalamajski and Oldberg, 2007). This resulted in the identification of critical residues (Fig. 3) and short synthetic peptides capable of specifically blocking SLRP-collagen interactions.

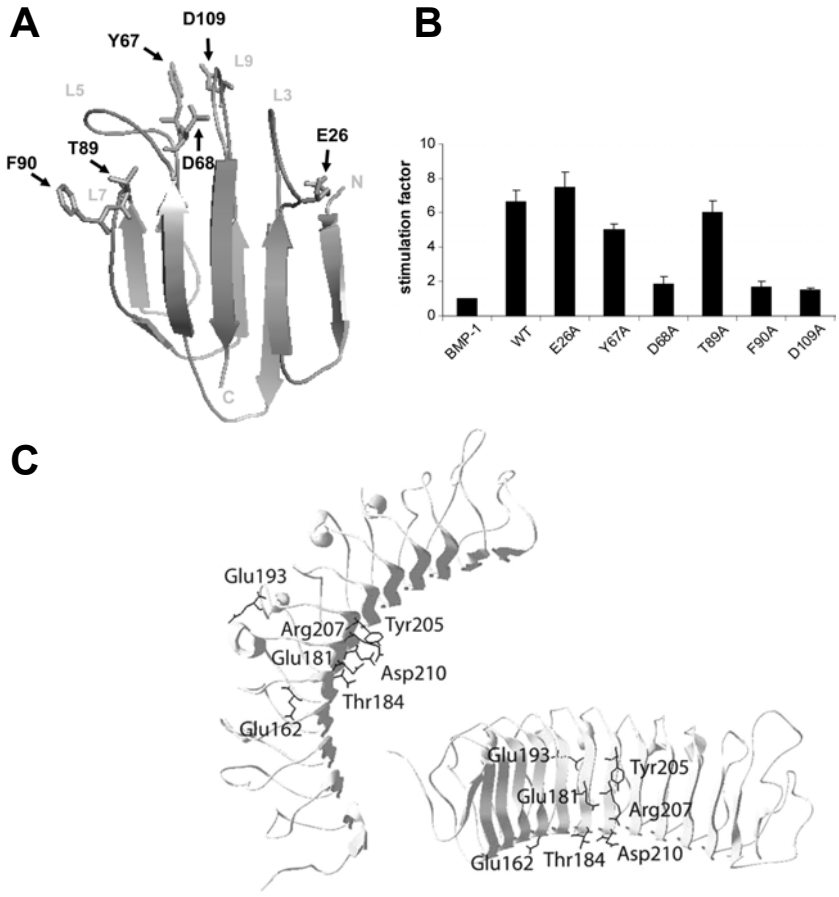

Figure 3. Interactions sites in PCPE-1 and decorin. (A) Molecular model of the CUB1 domain of human PCPE-1. (B) Effect of alanine mutations on the stimulation of BMP1 activity by PCPE-1 using a mini-procollagen III substrate, from Blanc et al., 2007. (C) Molecular modelling of the decorin molecule and identification of residues involved in interactions with collagen I, from Kalamajski et al., 2007.

3. Cells and clinical trials

The identification of stem cells has opened up enormous possibilities in the areas of tissue engineering and regenerative medicine. The hallmarks of stem cells are their capacity for self-renewal, their ability to differentiate into specialised cell types and their high proliferative potential. In the cornea, by far the most progress has been made with respect to

epithelial stem cells. The importance of the limbus (the zone between the cornea and the surrounding conjunctiva; Fig. 1) as a source of stem cells for corneal regeneration was first identified by (Kenyon and Tseng, 1989). This subsequently led to the clinical use of autologous cultures of adult corneal stem cells for permanent restoration of the corneal surface (Pellegrini et al., 1997; Rama et al., 2001). Typically, patients are victims of chemical or thermal burns, post-infective damage or other corneal pathologies (Steven-Johnson syndrome, cicatricial pemphigoid), where damage to the corneal surface leads to loss of stem cells and hence the inability to repair. As a consequence, the corneal surface becomes covered with conjunctival cells leading to vascularisation and loss of vision (Fig. 4A). Fortunately however in most cases only one eye is affected, so it is possible to culture autologous stem cells from the contralateral eye. In the procedure developed by participants in the project, cells from a small limbal biopsy are cultured *in vitro* to produce a new epithelial cell sheet which is used to replace the injured epithelium (Fig. 4B). In this way patients permanently recover their vision due to restoration of the stem cell stock. When damage to the cornea extends into the stromal layer (Fig. 4C), this requires a subsequent corneal graft from a donor following stem cell restoration (Fig. 4D). In the context of this project, one of the goals was to carry out multi-centric clinical trials in Italy (Venice, Milan) and France (Paris, Lyon). The trials conducted in Italy were very successful. Unfortunately however, it was not possible to obtain regulatory approval from the French authorities within the time frame available.

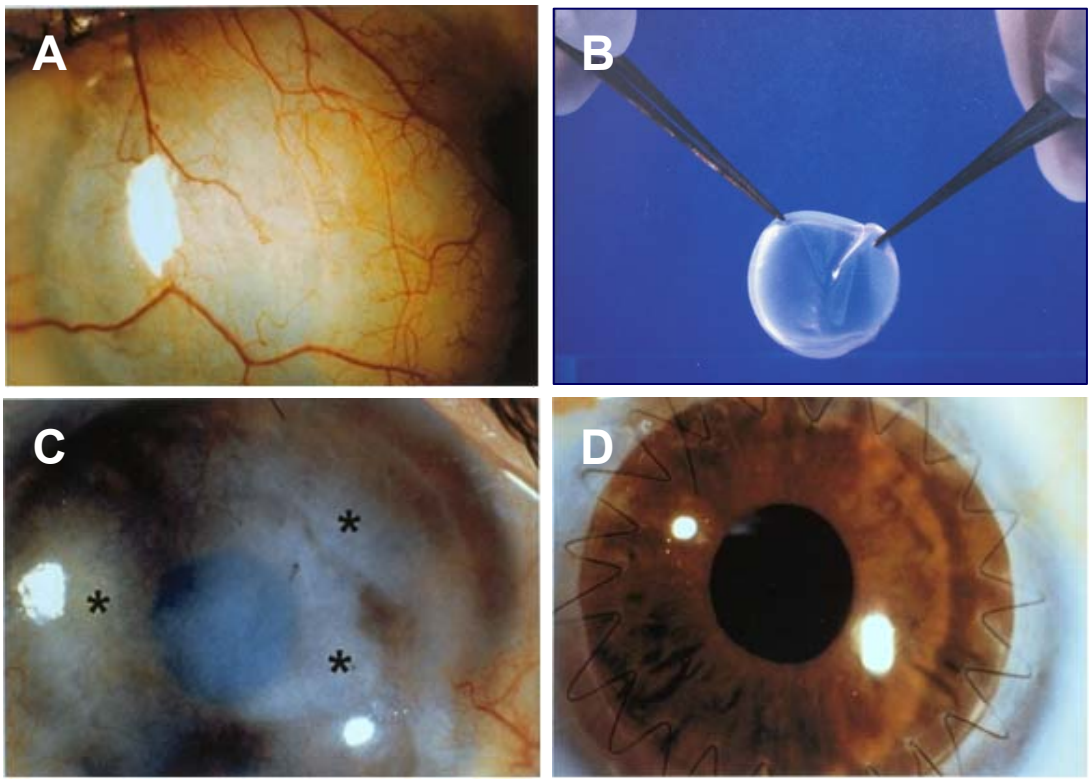

Figure 4. Restoration of the corneal surface using limbal stem cell derived epithelial cells. (A) Patient on admission showing conjunctivalization of the cornea. (B) Epithelial cell sheet prepared by in vitro culture of limbal stem cells. (C) Patient one year after restoration of epithelial stem cells. Note opaque regions (asterisks) indicating damage to the underlying stroma. (D) After grafting of donor cornea following restoration of epithelial stem cells. From Rama et al., 2001.

The molecular mechanisms by which stem cells retain their "stem-ness" are poorly understood. Several factors play a role, including cytokines, cell-cell contact and cell-matrix interactions, all of which contribute to the stem cell niche. One characteristic of stem cells is their relatively high expression of telomerase, the enzyme responsible for maintaining telomere length. During the course of the project, we showed that loss of proliferative potential of limbal stem cells is associated with a loss of telomerase activity and that increased expression of telomerase is sufficient to increase their lifespan (Pellegrini et al., 2004). We also identified the transcription factor ΔNp63α as a marker of limbal stem cells, as shown by its unique location in the basal layer of the limbus in resting corneas (Fig. 5A; from (Di Iorio et al., 2005)). When activated during corneal repair, limbal stem cells migrate towards the centre of the cornea and begin to express other ΔNp63 isoforms associated with regeneration and differentiation. More recently, we have identified two additional transcription factors, C/EBPδ and Bmi1, that are also associated with the limbal stem cells and which appear to be responsible for self-renewal, while ΔNp63 sustains proliferative potential (Fig. 5B; (Barbaro et al., 2007)).

The cells of the corneal stroma, called keratocytes, are normally relatively quiescent. Keratocytes secrete the components of the stromal extracellular matrix (collagens, SLRPs). Like epithelial cells, gene expression by keratocytes is strongly influenced by the microenvironment. Following wounding of the cornea, for example, keratocytes differentiate into myofibroblasts, leading to increased proliferation and altered matrix synthesis, notably down regulation of the SLRP keratocan. Such conditions can be mimicked in cell culture, where the keratocyte phenotype is maintained in the absence of serum while switching to serum-containing medium leads to myofibroblast differentiation. We have found that such wounding-associated keratocyte activation is also accompanied by the expression of the transcription factor VSX1 not normally expressed in cornea (Barbaro et al., 2006). Mutations in the VSX1 gene have been linked with corneal pathologies such as keratoconus and posterior polymorphous corneal dystrophy.

In view of this dependence on culture conditions, a necessary first step for tissue engineering applications was to find an optimum cell culture medium for preserving the keratocyte phenotype (Builles et al., 2006a). This was achieved by systematic analysis of different combinations of culture media and cytokines, screening for cell proliferation, maintenance of the keratocyte marker CD34 and absence of the myofibroblast marker α-smooth muscle actin. In further studies, with a view to identifying a possible stem cell source for human keratocytes, we also looked for variations in keratocytes isolated from different regions of the corneal stroma (Builles et al., 2008). Parameters measured were growth rate, number of population doublings, clonogenic potential and CD34 expression. Keratocytes were isolated from four regions: superior central, inferior central, superior peripheral and inferior peripheral. It was found that cells from the superior peripheral region had the highest proliferative and clonogenic potentials, while CD34 expression was somewhat higher in the central region. Though keratocyte stem cells were not formally identified, the high proliferative potential (up to 40 population doublings) of the cells isolated made them suitable for tissue engineering.

The innermost layer of the cornea, the endothelium, consists of a monolayer of endothelial cells whose main function is to prevent swelling due to water uptake and consequent loss of transparency. Endothelial cells proliferate slowly and frequently suffer from losses during storage of donor corneas (Builles et al., 2006b). To date, it has not been possible to identify an adult stem cell source of human corneal endothelial cells. Preliminary results (Bednarz et al, unpublished observations) indicate that endothelial cells with a relatively high proliferative potential can be isolated from the peripheral region of the cornea. These cells have been used to establish a bank of human endothelial cells, either for

restoration of the endothelial cell layer in donor corneas or for use in corneal tissue engineering.

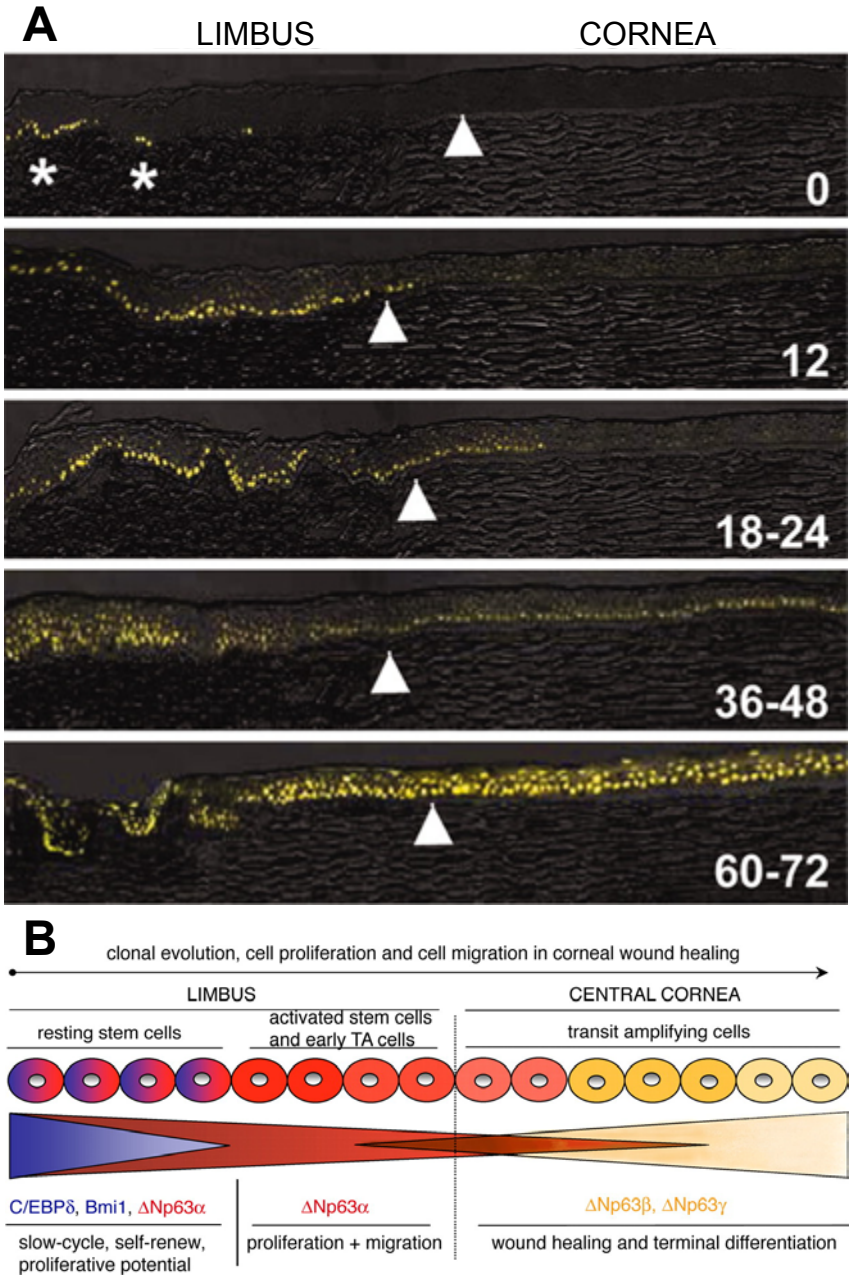

Figure 5. (A) Expression of p63 in resting corneas (0 hrs) after activation in culture for up to 72 hrs. p63 isoforms were identified by the 4A4 monoclonal antibody. At time 0, clusters of p63+ cells (asterisks) were observed only in the limbal basal layer. Arrows indicate the limbus-cornea border. From Di Iorio et al., 2005. (B) Model for regeneration of the human corneal epithelium, from (Barbaro et al., 2007). C/EBPδ, Bmi1 and ΔNp63α maintain the resting stem cell state. Following activation, C/EBPδ and Bmi1 expression is switched off leading to proliferation and migration. Stem cells then differentiate to transit amplifying cells leading to expression of other ΔNp63 isoforms.

4. Cell-matrix interactions

It is clear that interactions between corneal cells and the surrounding extracellular matrix are important for the regulation of cell differentiation, cell death (apoptosis) and cell

migration. Part of the project was therefore devoted to the study of these interactions and their consequences for cell behaviour.

Heparan sulphate proteoglycans (syndecans, glypicans) are ubiquitous cell surface components. Many extracellular matrix molecules bind to heparin, a close analogue of heparan sulphate, thereby providing a widespread mechanism of cell-matrix interactions. One such molecule is type V collagen, an important component of corneal collagen fibrils that helps to maintain the narrow fibril diameters required for optical transparency. The heparin binding site in collagen V has been localised to the α1(V) chain. During the course of the project, this interaction was characterised in detail using synthetic peptides and surface Plasmon resonance technology (Ricard-Blum et al., 2006). It was found to be cation-dependent and greatly enhanced by assembly into a collagen triple-helical structure. Heparan sulphate-dependent cell surface interactions were also identified for ADAMTS2 (Colige et al, unpublished observations) and PCPE1 (Kessler et al, unpublished observations).

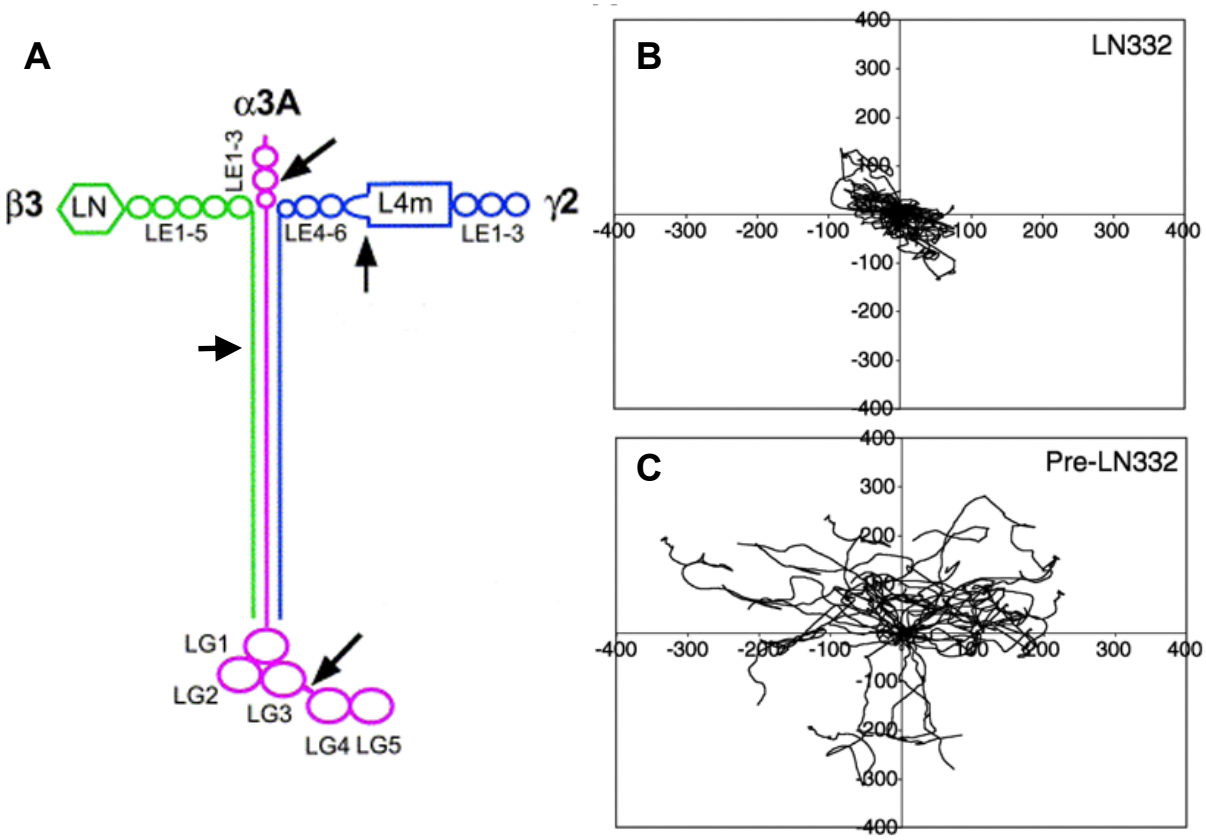

Figure 6. (A) Structure of the laminin 332 molecule, showing the three polypeptide chains (α3,β3,γ2), LE and LG domains and proteolytic cleavage sites (arrows). (B) Tracks of NHK cells migrating on (B) mature laminin 322 (without LG4-LG5 domains) or (C) precursor laminin 322 (with LG4-LG5 domains) showing increased cell motility when the LG4-LG5 domains are intact. From Bachy et al., 2008.

Laminins are major structural proteins of basement membranes and also signalling molecules that interact with cell surface receptors such as integrins and syndecans, thereby transmitting morphogenetically important information to the cell interior. Laminin 332 (formerly known as laminin 5) is found in the basement membrane of specialised epithelia such as the cornea where it plays an essential role in adhesion, migration and repair. The molecule consists of three polypeptide chains (Fig. 6), all of which are subject to proteolytic attack. The C-terminal region of the α3 chain, including the LG4/5 fragment that is absent in mature laminin 332, is involved in binding to cell surface receptors. We have shown that cell

surface binding of precursor laminin 322 involves both the integrin α3β1 and syndecan-1 receptors, while only syndecan-1 is involved in binding to LG4/5 (Bachy et al., 2008). In contrast, mature laminin 332 binds to both α3β1 and α6β4/β1 integrins, suggesting that the binding site for the latter is hidden in precursor laminin 332. These interactions may account for the observation that cells show reduced adhesion and increased syndecan-mediated migration on precursor laminin 332 compared to mature laminin 322 (Fig. 6). Finally, we have also identified the β3 chain as a substrate for matrix metalloprotease 7 (Remy et al., 2006). In contrast to the α3 chain however, proteolytic cleavage of the β3 chain leads to increased cell migration. These observations give insights into the role of laminin 322 during epithelial outgrowth and migration at the wound healing front.

In addition to laminin 332, laminin 511 (formerly laminin 10) is also present in corneal and conjunctival basement membranes. Cell adhesion to laminin 511 is mediated both by integrin α3β1 and by the Lutheran cell surface glycoprotein. We have found Lutheran to be expressed in the basal cells of the human corneal epithelium (Hasenson et al., 2005). In addition, we have observed the expression of laminin 111 during human embryonic development of the cornea, both in the corneal epithelium and in Descemet's membrane (Bystrom et al., 2006). Finally, corneal scarring associated with keratoconus or deep lamellar keratoplasty was found to lead to ectopic expression of laminin chains and in particular laminin 332 in Descemet's membrane, where normally it is absent (Bystrom et al., 2007).

5. Biomechanics, tissue engineering and animal testing

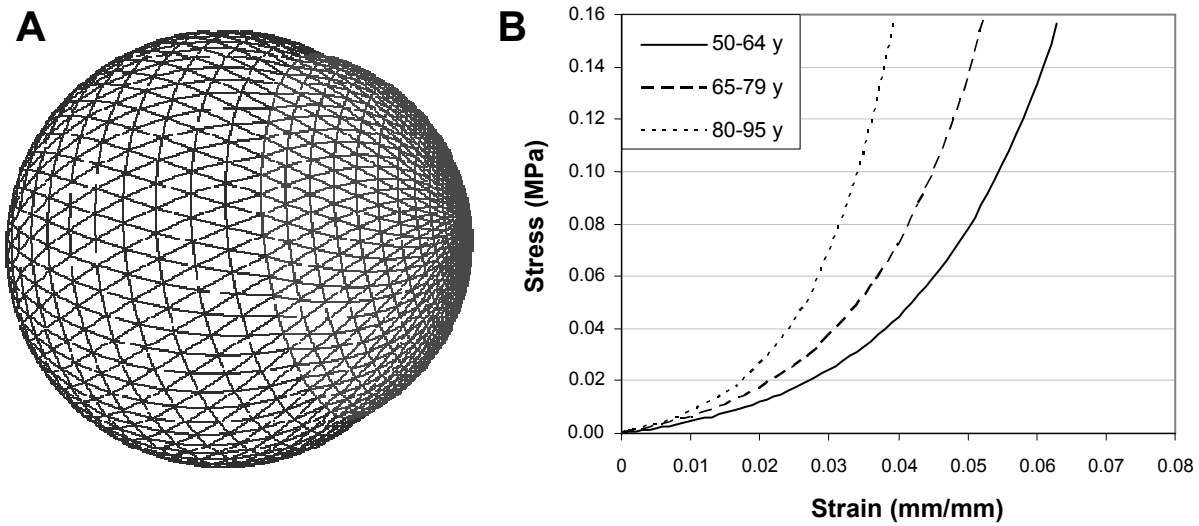

Figure 7. (A) Nonlinear finite element mechanical modelling of the entire eye globe, with the cornea on the right. (B) Age-dependent increase in corneal stiffness, from Elsheikh et al., 2007a.

In order to reconstruct a cornea with the required biomechanical properties, it is important to understand the biomechanics of normal corneas. To do so, we devised a non-linear finite element model of the cornea (Fig. 7A) to simulate the effects of central corneal thickness, radius of curvature and material properties on the determination of intraocular pressure (IOP) using standard Goldmann aplanation tonometry (Elsheikh et al., 2006). It was found that variations in central corneal thickness and material properties can lead to errors in the estimation of IOP, which could have important consequences in the diagnosis of glaucoma. In further studies, by inflation testing, we found that the mechanical stiffness of the cornea increases with age (Fig. 7B). This may be a result of age-related changes in collagen cross-linking due to the accumulation non-enzymatic glycation end-products and may itself

increase the risk of glaucoma due to an increase in IOP (Elsheikh et al., 2007a; Elsheikh et al., 2007b).

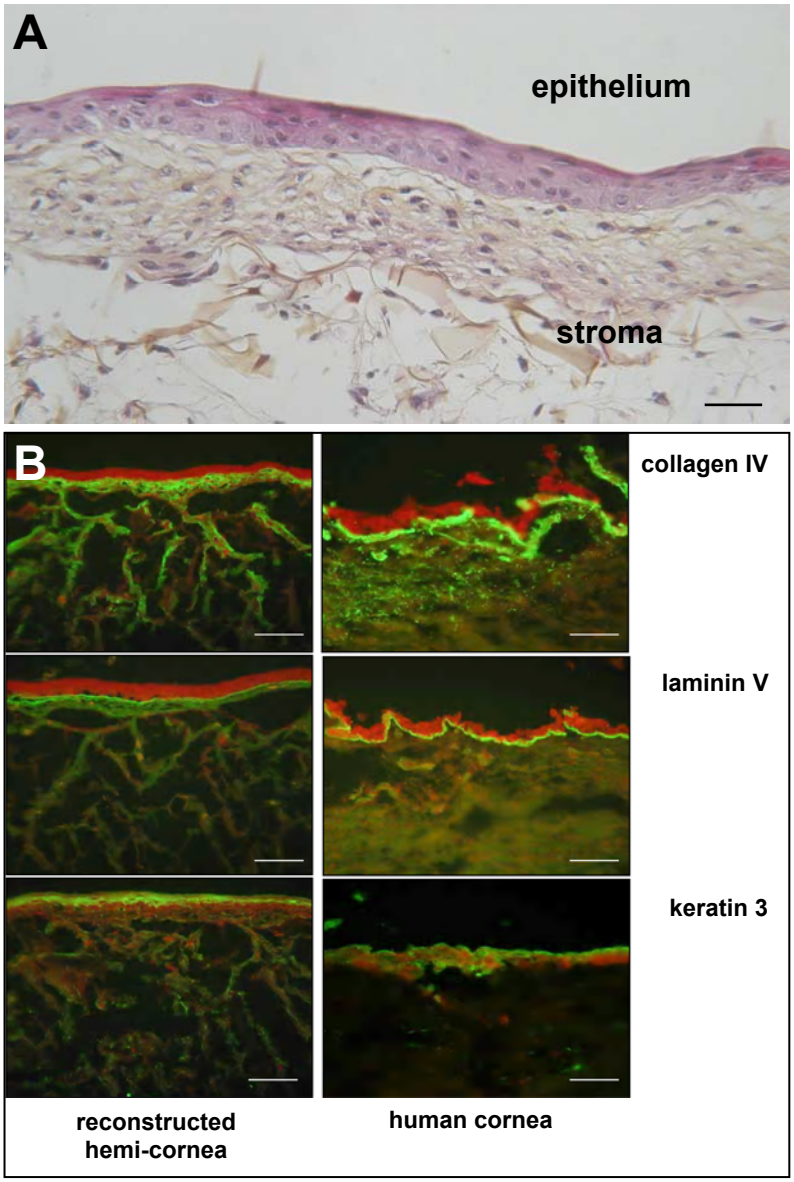

Figure 8. Reconstructed human hemi-cornea. (A) Stained with hematoxylin–phloxin–saffron. Scale bar 50 µm. (B) Immunostained for basement membrane (collagen IV, laminin 5) and epithelial cell (keratin 3) markers. Scale bars 100 µm. From Builles et al., 2007a.

For tissue engineering of the cornea, the first objective was to reconstruct a hemi-cornea (epithelium + stroma) for use an *in vitro* alternative to animal models for pharmacotoxicity testing. This follows recent European legislation (2003/15/EC) banning the use of animals for testing of cosmetic products, and in particular the Draize test carried out on rabbit corneas which, besides causing considerable discomfort and pain, is not always predictive of the human response. A number of *in vitro* epithelial cell cultures have been commercialised for *in vitro* testing, but so far none of these incorporate a stromal or endothelial cell layer. This and the fact that some models rely on the use of immortalised cells make them poorly representative of the *in vivo* situation. In order to devise a more physiological model, we reconstructed a hemi-cornea using human adult stem cell derived keratinocytes (epithelium) and human keratocytes (stroma) (Builles et al., 2007a). For the stroma, cells were supported

by a three-dimensional porous foam-like scaffold previously developed for skin reconstruction composed of collagen, glycosaminoglycan and chitosan. As shown in Fig. 8, the resulting hemi-cornea included of a well differentiated stratified epithelium and basement membrane. Furthermore the stromal keratocytes synthesized an extracellular matrix containing collagens I, V and VI, as in native corneas. The presence of the epithelium was found to have a significant effect on the organisation of the extracellular matrix in the stroma, as shown by the diameters of the newly synthesized collagen fibrils which were closer to physiological values than in the absence of epithelium (Builles et al., 2007b). The reconstructed hemi-cornea is currently undergoing validation for use in pharmacotoxicity testing.

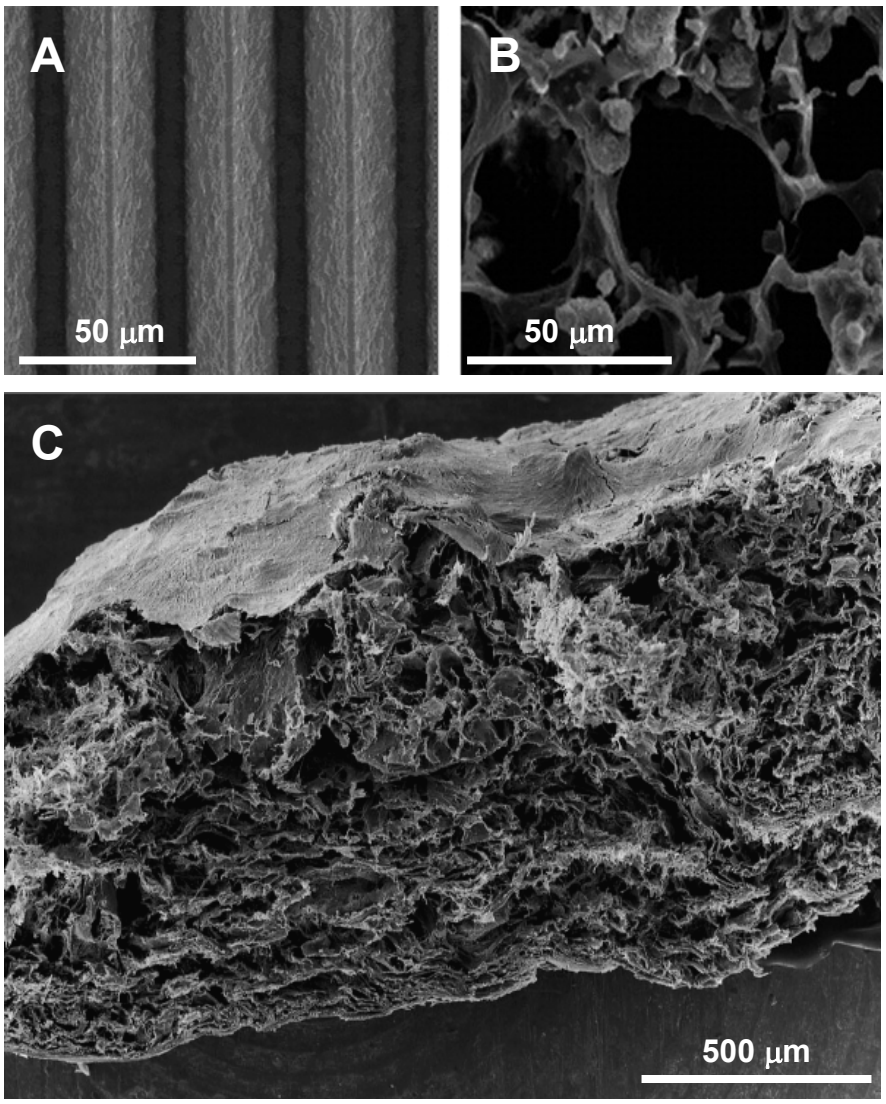

Figure 9. Micropatterned films and foams. (A) Micropatterned film and (B) foam-like scaffolds made from PHBV/P(L/DL)LA polyester mixtures, from Zorlutuna et al., 2006. (C) Cross-linked collagen foam populated with human keratocytes, from Vrana et al., 2007a.

In addition to the collagen-GAG-chitosan scaffold described above, a variety of other scaffolds were tested. These included scaffolds made from mixtures of the naturally occurring and synthetic polymers poly(hydroxybutyric acid-*co*-3-hydroxyvaleric acid) (PHBV) and poly(l-lactide-*co*-d,l-lactide) (P(L/DL)LA), respectively (Zorlutuna et al., 2006). These were prepared either in the form of micropatterned films or foams (Fig. 9A,B), as supports for

D407 epithelial cells and 3T3 fibroblasts. The micropatterned films were made using silicon templates produced by photolithography. Both types of scaffold supported cell growth and the micropatterned films led to cell alignment. In subsequent studies (Zorlutuna et al., 2007), PHBV/P(L/DL)LA films were coated with corneal keratocytes or D407 epithelial cells and the effects of cell growth on the mechanical properties of the films were measured. It was found that film strength increased with time in culture when coated with keratocytes. In contrast, with uncoated or D407 cell coated films, mechanical strength decreased with time. This was attributed to the ability of corneal keratocytes to stabilise the films by the formation cell-cell contacts and extracellular matrix.

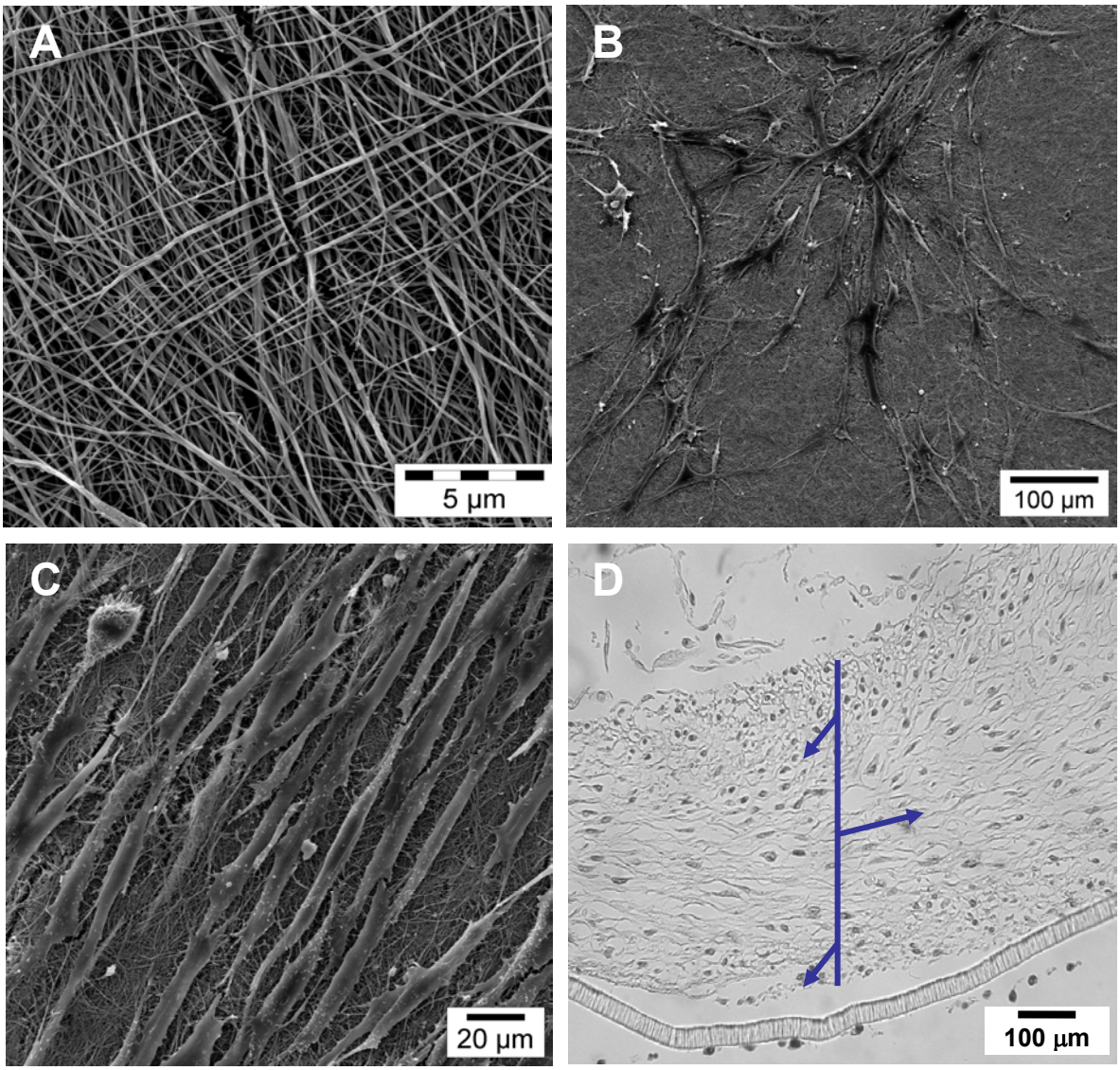

Figure 10. Corneal stroma reconstructed using a magnetically oriented orthogonal collagen fibril scaffold. (A) Two orthogonal layers visualised by scanning electron microscopy. Human keratocytes cultured on (B) non-oriented and (C) oriented collagen supports. (D) Histological section showing penetration of keratocytes into a three-layer orthogonal stack showing cells aligned along the initial directions of the collagen fibrils (indicated by arrows). From Torbet et al., 2007.

Micropatterned films were also prepared as above but with EDC/NHS cross-linked collagen type I (Vrana et al., 2007b). As with the polyester films, when coated with corneal keratocytes, the mechanical strength of these films also increased with time, unlike uncoated films or films coated with D407 epithelial cells. This correlated with the production of a newly synthesized extracellular matrix by the keratocytes thereby reinforcing the scaffold. Furthermore, micropatterned films appeared to help maintain optical transparency when coated with keratocytes (Vrana et al., 2008). In complementary studies, the behaviour of corneal keratocytes in EDC/NHS cross-linked collagen foams was also studied (Vrana et al., 2007a). Keratocytes were found to penetrate into the foam (Fig. 9C) and produced a newly synthesised extracellular matrix producing collagens I, V and VI. Finally, cross-linked collagen foams incorporating chondroitin sulphate were used to reconstruct a full-depth cornea using human keratocytes, adult stem cell derived epithelial cells and immortalized human endothelial cells (Vrana et al, submitted for publication).

Finally, we developed a new technology to reconstruct the plywood like structure of the corneal stroma, using strong magnetic fields to orient collagen fibrils in successive layers (Torbet et al., 2007). Collagen molecules in cold acid solution spontaneously assemble into fibrils when the pH and temperature are adjusted to physiological conditions. If fibril assembly is carried out in a strong magnetic field, the fibrils become uniformly aligned perpendicular to the field direction. Once formed, the orientation of the fibrils remains fixed. This means that orthogonal stacks of fibrils can be built up layer by layer simply by rotating the sample by 90° at each step. In this way, we reconstructed an artificial stroma which mimicks very closely its natural counterpart (Fig. 10, compare with Fig. 1C). We found that transparency of the reconstructed stroma could be improved by the addition of SLRPs. In cell culture, human corneal keratocytes were found to align along the fibril direction (Fig. 10B,C). Furthermore, in multilayer stacks, keratocytes penetrated the entire thickness whilst preserving the orientations of the individual layers (Fig. 10D). Such orthogonal stacks could therefore have applications in corneal stromal repair. As a first step in this direction, these stacks were implanted in rabbit corneas. Preliminary results are encouraging (unpublished observations).

6. Conclusions

The project has been largely successful, having achieved most of the aims initially set out. We have developed new protocols for the production of recombinant extracellular matrix proteins and have gained new insights into the regulation of extracellular enzymes and the roles of cell-matrix interactions in corneal structure and repair. We have also identified new molecular mechanisms controlling the phenotype of limbal stem cells and have shown that keratocytes from different regions of the cornea vary in proliferative potential. Successful clinical trials on restoration of limbal stem cell deficiency have been done. We have developed a hemi-cornea using normal human cells for applications in pharmacotoxicity testing, currently undergoing validation. Finally, we have devised new technologies for the construction of three-dimensional scaffolds and shown these to be useful for corneal tissue engineering, including pre-clinical testing in animal models.

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