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<th>Cell sheet technology meets macromolecular crowding: The self-assembly approach for corneal stromal development</th>
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Cell Sheet Technology Meets Macromolecular Crowding:
The Self-Assembly Approach For Corneal Stromal Development

A Thesis Submitted to the National University of Ireland for the
Degree of Doctor of Philosophy

By
Pramod Kumar

Research Supervisor: Dr Dimitrios Zeugolis

January 2015

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<td>CR</td>
<td>75µg/ml Carrageenan</td>
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<tr>
<td>DxS</td>
<td>100µg/ml Dextran sulfate 500kDa</td>
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<tr>
<td>FC</td>
<td>25mg/ml Ficoll®400 kDa + 37.5mg/ml Ficoll®70kDa</td>
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<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
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<td>65% poly(N-isopropyl acrylamide) / 35% poly(N-tert-butyl acrylamide)</td>
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Acknowledgements

A project work does not complete without the support and heartfelt wishes of numerous people. As the present project has acquired this final structure, memories unreel in my mind, reminding me the ups and down confronted during this tenure. I learned that the only way we are going to get anywhere in life is to work hard. Problems can become opportunities, when the right people come together. This is the correct time to say thanks for the timely help of people, who showed me direction and guidance to achieve my aim. This is not just a formal thank; it is rather a deep gratitude, which comes from my heart.

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people gave me the courage to attempt something great and I could not have gone as far as I did without their support. These formal words of acknowledgement would hardly justify my feelings, respect, and gratitude for their love, care and support.

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Pramod Kumar
Abstract
Therapeutic strategies based on the principles of tissue engineering by self-assembly put forward the notion that functional regeneration can be achieved by utilising the inherent capacity of cells to create highly sophisticated supramolecular assemblies. However, in dilute ex vivo microenvironments, prolonged culture time is required to develop extracellular matrix-rich implantable devices. Herein, we assessed the influence of macromolecular crowding, a biophysical phenomenon that regulates intra- and extra-cellular activities in multicellular organisms, in human corneal fibroblast culture. In the presence of neutral macromolecules, abundant extracellular matrix deposition was evidenced as fast as 48 hours in culture, even at low serum concentration. The addition of negative charged galactose derivative (carrageenan) in human corneal fibroblast culture, even at 0.5% serum, increases by 12-fold tissue-specific matrix deposition, whilst maintaining physiological cell morphology and protein / gene expression. Gene analysis indicates that a glucose derivative (dextran sulphate) may drive corneal fibroblasts towards a myofibroblast lineage. Temperature responsive copolymers allowed the detachment of dense and cohesive supramolecularly assembled living substitutes within 6 days in culture, with morphological and histological properties similar to native tissue. Collectively, these results indicate that macromolecular crowding may be suitable not only for clinical translation and commercialisation of tissue engineering by self-assembly therapies, but also for the development of in vitro pathophysiology models.
Chapter 1

Introduction
1.1. Cellular and extracellular composition of the corneal stroma

The cornea is outermost transparent window of the eye. The mammalian cornea tissue (Figure 1.1) is composed of three cellular layers: the outer multi-layered epithelium that protects the tissue from injury and foreign matter; the middle stromal layer, consisting of rich extracellular matrix (ECM) and interspersed keratocytes, which is responsible for the transparency and mechanical stability of the tissue; and the inner single layered endothelium, which maintains the hydration of the tissue. The two non-cellular layers of cornea, the basement and the pre-Descemet’s / Descemet’s membranes, connect the epithelium-stromal and the stromal-endothelium layers respectively and facilitate exchange of biological signals and nutrients [1].

Figure 1.1. The morphology of human cornea.

Corneal keratocytes, corneal fibroblasts, corneal myofibroblasts and corneal stromal stem cells are the various cellular population of the human corneal stromal layer (Table 1.1). Corneal keratocytes are quiescent, dendritic cells, which, upon injury to the cornea, stimulated to repair phenotypes or undergo apoptosis [2-4]. Primary keratocytes express CD34 and aldehyde dehydrogenase (ALDH3) and secrete keratan sulphate proteoglycans (PGs). Keratan sulphate (sulphated poly-N-
(acetyllactosamine repeats) is predominant glycoaminoglycan present in corneal stroma which regulates the collagen fibril diameter; its expression in keratocytes is further enhanced during pathology to regulate the homeostasis [5]. These markers are reduced during keratocytes differentiation into fibroblasts and myofibroblasts, whilst collagen type I and type III are increased [5]. Keratocytes become fibroblast-like cells during in vitro culture and are characterised by flat, spindle shape morphology and are producing high amounts of ECM during the wound repair mechanism. The corneal fibroblasts may act as mediators of inflammation and play an active role in various corneal diseases and wound healing in conjunction with epithelium [6]. Long-term exposure of corneal keratocytes or corneal fibroblasts to serum results in polygonal myofibroblast differentiation. The myofibroblast are representative of scar tissue, with the highest synthesis of collagenous matrix and very low, if any cell division [7]. Further, myofibroblasts express high levels of alpha smooth muscle actin (α-SMA), biglycan, and the extra domain A (EDA or EIIIA) of cellular fibronectin. Chondroitin sulphate / dermatan sulphate glycosaminoglycans (GAGs) abundance, chain length and sulphation are increased as keratocytes differentiate into fibroblasts and myofibroblasts, whilst hyaluronan, absent in keratocytes, is secreted by fibroblasts and myofibroblasts. Keratan sulphate synthesis, chain length and sulphation are reduced in fibroblasts and myofibroblasts differentiated cells [8]. The presence of other stromal cell population; the multipotent corneal stromal stem cells are also reported in literature, which express the ABCG2/ PAX6 and devoid of epithelium marker keratin 12. These stromal stem cells can be differentiated to corneal keratocytes phenotype in vitro with high expression of keratocytes markers including keratocan, ALDH3A1, and keratan sulphate [9].

The stromal layer constitutes the 90% of the tissue and is composed of various collagens, GAGs and PGs assembled in a hierarchical and tissue-specific fashion (Figure 1.2) [10]. The dense and closely packed small diameter (25-35 nm) collagen fibrils are arranged homogenously throughout the stromal layer in the form of orthogonal layers (lamellae), in which around 200 collagen fibrils are arranged parallel to each other and perpendicular to the adjacent lamellae [11, 12]. This
specific spatial arrangement of collagen fibrils provides mechanical strength and transparency to the tissue [13]. According to Maurice theory, the lattice arrangements of the fibrillar collagen in the stroma is responsible for the transparency [14], whilst the Goldman and Benedik theory proposes that the spacing of the neighboring collagen fibrils in stroma is responsible for the transparency of the tissue [15]. Type I collagen is the major structural component and is co-distributed with collagen type V, which regulates fibril assembly and diameter [16]. The corneal stroma also contains collagen type VI, which forms micro-fibrillar structures; the presence of fibril associated collagen type XII and type XIV; and the non-fibrillar collagen type XIII and type XVIII is also reported in corneal stroma. Although in small amounts, these collagens are important in structure and function of cornea; for example, collagen type VI and collagen type XII are responsible for the stability of collagen fibrillar array; collagen type XIV is responsible for the compaction of cornea; collagen type XXIV regulates early fibril diameter [12]. Small amount of collagen type III is also present in cornea during pathology, such as inflammation or wound healing and in vitro culture, indicative of its involvement in the wound healing process [17-19]. Collagen type IV (component of basement membrane) is found adjacent to the corneal stromal and in basement membranes. It has important role in attachment of cells to the basement membrane, cell migration, proliferation and differentiation in coordination with Laminin [20, 21]. The role of collagen type IX and collagen type XVII in mature cornea is unclear which are also reported in corneal stroma during the developing stage [19].

Chondroitin sulphate / dermatan sulphate, keratan sulphate and heparan sulphate are the GAGs identified in corneal stroma. Keratan sulphate (e.g. lumican, keratocan, mimecan) and chondroitin sulphate / dermatan sulphate (e.g. decorin, biglycan) proteoglycans are the major proteoglycans present in cornea, whilst heparan sulphate proteoglycans are minor components of the cornea and are synthesised mainly by epithelial cells [2]. Lumican and decorin regulate collagen fibril growth and spacing between collagen fibrils, contributing that way to corneal transparency [22-25]. Increase in chondroitin sulphate / dermatan sulphate proteoglycans (e.g. decorin, biglycan) is associated with chronic corneal pathologic conditions [26], such as
congenital stromal corneal dystrophy [27]. In macular corneal dystrophies, keratan sulphate and chondroitin sulphate / dermatan sulphate chain size is reduced; the concentration of keratan sulphate chain reduced; the concentration of chondroitin sulphate / dermatan sulphate chain is increased; and hyaluronan, not normally present in healthy adult corneas, is detected [28].

Figure 1.2. Structure of human corneal stromal tissue.
Table 1.1. | Characteristics of human corneal keratocytes, fibroblasts, myofibroblasts and stromal stem cells.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Keratocytes</th>
<th>Fibroblasts</th>
<th>Stem Cells</th>
<th>Myofibroblasts</th>
</tr>
</thead>
</table>
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1.2. Therapeutic approaches towards corneal repair and regeneration

Injuries (e.g. burns) [30], infections [31] and degenerative conditions, congenital or acquired in nature (e.g. Conjunctivitis, Stevens-Johnson syndrome, Fuchs' dystrophy, Keratoconus) [32] may impair corneal function and, in severe cases, lead to blindness [33]. In fact, over 2 million new cases of blindness are recorded annually worldwide [34], imposing the need for development of functional therapeutic strategies. Over the years, significant advancements have been achieved in the area of corneal repair and regeneration (Table 1.2) and, to date, penetrating keratoplasty, keratoprosthesis, amniotic membrane and bioengineering approaches constitute our toolbox in fighting blindness.

Table 1.2. | Historic progress in therapeutic approaches for the treatment of corneal diseases.

<table>
<thead>
<tr>
<th>Therapeutic Development</th>
<th>Year</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Use of glass as a corneal substitute</td>
<td>1789</td>
<td>[35]</td>
</tr>
<tr>
<td>Pig cornea transplantation to human (USA)</td>
<td>1838</td>
<td>[36]</td>
</tr>
<tr>
<td>First experimental corneal implantation</td>
<td>1853</td>
<td>[37]</td>
</tr>
<tr>
<td>Use of quartz as a corneal substitute</td>
<td>1853</td>
<td>[38]</td>
</tr>
<tr>
<td>First successful corneal transplantation</td>
<td>1906</td>
<td>[39]</td>
</tr>
<tr>
<td>First eye bank</td>
<td>1944</td>
<td>[40]</td>
</tr>
<tr>
<td>First artificial cornea made up of PMMA</td>
<td>1947</td>
<td>[41]</td>
</tr>
<tr>
<td>First full thickness cornea using the native cells</td>
<td>1993</td>
<td>[42]</td>
</tr>
<tr>
<td>First successful autologous graft transplantation</td>
<td>1997</td>
<td>[43]</td>
</tr>
<tr>
<td>First full thickness human cornea</td>
<td>1999</td>
<td>[44]</td>
</tr>
<tr>
<td>First successful corneal epithelium cell sheet transplantation derived from cell sheet technology</td>
<td>2004</td>
<td>[45]</td>
</tr>
<tr>
<td>Scaffold free full thickness cornea</td>
<td>2010</td>
<td>[46]</td>
</tr>
<tr>
<td>Stromal cell sheet</td>
<td>2013</td>
<td>[47]</td>
</tr>
</tbody>
</table>
1.2.1. Penetrating keratoplasty (corneal grafting)
Penetrating keratoplasty is the replacement of full thickness diseased tissue with the healthy donor tissue and is considered the gold standard in clinical practice, since the first successful corneal transplantation in 1905 by Zirm [39]. In combination with autologous limbal and conjunctival epithelial cells, the grafts result in full corneal epithelisation within 7 days after surgery in patients with severe chemical or thermal burns [48]. However, corneal grafting is far from optimal. Despite the immuno-privileged nature of the cornea tissue [49, 50], only 50% of the patients do not need immuno-suppression whilst tissue vascularisation and high inflammation frequently lead to allograft rejection [51-53]. The success of corneal transplantation also depends on the disease / injury; a 35% success rate has been reported in bullous keratopathy, whilst patients suffered of alkali burns have very little chance of success [54, 55]. Preliminary xeno-transplantation (pig cornea) data are positive [56], but we are still far away from a functional therapy. Indeed, the demand of healthy donor tissue exceeds by far the availability [57]. Every year, around 10 million new people are affected by various eye disorders and most of them may benefit from corneal transplantation [54]. In US alone, over 50,000 tissue graft surgeries take place annually with almost 31% annual increase [58].

1.2.2. Keratoprosthesis
Keratoprosthesis is the replacement of damaged or diseased tissue with an artificial implantable device [59]. The first transplantation was performed by Nussbaum in late 1800, using a quartz crystal [37]. Several synthetic [e.g. poly(methyl methacrylate), poly(tetrafluoroethylene), poly(urethane), poly(2-hydroxyethyl methacrylate), Teflon] devices have been accessed over the years, however, they have achieved limited success in clinical setting [60-63]. Primary limitations include complex surgery; high risk of vision-threatening complications (e.g. late melting of anterior corneal lamella); postoperative complications (e.g. retro-prosthetic membrane, persistent epithelial defects); and improper integration into the host tissue [64].
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1.2.3. Amniotic membrane
The use of amniotic membrane in ophthalmic diseases was first demonstrated by de RÖTTH in 1940 [65]. The amniotic membrane is composed of the inner part of the placental layer, a basement membrane and an avascular stromal layer. Amniotic membrane has been used effectively in clinical setting for several pathologic conditions, including: management of persistent epithelial defects; corneal ulcers; bacterial keratitis; descemetocele formation; corneal perforation; limbal stem cell deficiency; symptomatic bullous keratopathy; band keratopathy; chemical or thermal injury; repair of conjunctival defects; pterygium surgery; bleb repair; scleral perforation; Stevens-Johnson syndrome; high-risk corneal transplantation; and for inhibition of inflammation and fibrosis of the cornea [66-70]. Amniotic membrane is used in the form of a temporary patch or permanent graft in contact with corneal cells or conjunctival cells and is commercially available in cryo-preserved form with the epithelium (AmnioGraft®, ProKera™) or in de-epithelised dehydrated form (AmbioDry™, AmbioDisk™, TutoPlast®). Amniotic membranes have also been used in conjugation with various cell populations with good results in vitro, in preclinical models and in clinic. For example, amniotic membrane facilitated phenotype maintenance of corneal keratocytes, as evidenced by downregulation of transforming growth factor-β (TGF-β); upregulation of keratocan; and maintenance of the dendritic morphology of the cells, even in the presence of serum [71, 72]. Further, human corneal endothelium cells grown on amniotic membrane were uniform in shape, formed tight cell junctions and induced minute oedema and maintained their thickness and transparency after transplantation in a rabbit model [73]. Limbal epithelial cells delivered using an amniotic membrane promoted corneal re-epithelisation and prevented conjunctivitis and neovascularisation in rabbits with limbal stem cell deficiency [74]. When limbal epithelial cells were cultured and expanded on amniotic membrane and implanted in the damaged eye of patients with severe unilateral corneal disease after superficial keratectomy to remove fibrovascular ingrowth, results in complete re-epithelisation of the corneal surface within two to four days of transplantation. The ocular surface was covered with corneal epithelium and the clarity of the cornea was improved within a month.
of post-implantation; and no recurrent neovascularisation or inflammation was detected up to 15 months post-implantation [75]. Despite the positive clinical data to date, amniotic membranes are far from optimal. Indeed, amniotic membrane implantation has been associated with bacterial, fungal and viral infections [76]. Other limitations of amniotic membrane use include inter- and intra- donor batch variation, unclear mode of action, tardy methods of processing and preservation and lack of defined standardisation criteria [77, 78]. The use of amniotic membrane has also been associated with corneal calcification [79], granulomatous reaction [80], sub-membrane haemorrhage and early detachment of the membrane after transplantation [80, 81], further hindering their use in clinical practice.

1.2.4. Bioengineered approach

Given the limited clinically acceptable outcome of the traditional approaches, corneal bioengineering has the potential to help millions of patients awaiting suitable donor tissue, given the recent inter- and multi- disciplinary advancements [82]. Bioengineering approaches are based on natural [83-86] or synthetic [29, 87-89] scaffold combined with appropriate biologics / therapeutics molecules and cell population(s). The ideal bioengineered substitute for cornea should offer several specific features, including transparency, refraction and mechanical stability. The scaffold material provides the required structural support for cell attachment, growth, migration and subsequent tissue development [90-92]. Although scaffold-based therapies have demonstrated acceptable levels of cell adhesion, proliferation and differentiation; suitable mechanical stability; and optical transparency, they fail to mimic the natural microenvironment of the native complex corneal tissue [93], resulting in serious post-operative complications, including hyper-acute or delayed rejection [94, 95]; immunological responses [85]; foreign body response [96]; neither stable nor clear corneal epithelisation [97]; suboptimal retention [98]; and inflammation [99]. Thus, it was suggested that a more effective approach is needed to promote tissue repair [90], which stimulated the development of scaffold-free tissue engineering [100] / cell sheet tissue engineering [101] / modular tissue engineering [102, 103] / tissue engineering by self-assembly [104, 105]. Such
approaches require removal of cells from their highly dynamic environment and expansion *in vitro* to attain adequate numbers, which will be subsequently transplanted either as cell suspension or cellular constructs / cell sheets. Such approaches offer significant advantages over conventional therapies, including ability to deliver a complex mixture of physiologically relevant biological factors and signals with supreme regenerative capacity and ability to avoid immunogenic or inflammatory responses that may occur using allografts and scaffolds [104, 106, 107]. Corneal epithelium deficiency has already been treated in clinical setting using autologous mouth mucosal epithelium cells into a multilayer cell sheet format [45]. Further, human autologous corneal epithelial sheets, generated by serial cultivation of limbal cells, have been shown to improve the corneal transparency in patients suffered of severe alkali burns comfort and visual acuity [43]. A transparent stromal layer with adequate mechanical properties has also been developed using primary human corneal stromal cells and SV40-immortalised human corneal keratocytes [47]. Human corneal stem cells have been shown to secrete and produce a corneal-like stromal tissue *in vitro* [108]. Using aligned fibrous substrates, a more clinically relevant stromal layer was produced using human corneal stromal stem cells than that of human corneal fibroblasts, as the human corneal fibroblasts tended to differentiate towards myofibroblasts and deposited a less organized ECM, proportional to corneal scarring [109]. Further, human corneal stromal stem cells have been shown great potential for the regeneration of stromal layer in a murine model [110]. Human corneal endothelial cell sheets have also been produced with similar to *in vivo* cellular characteristics (e.g. hexagonal cell shape, numerous microvilli and cilia, abundant cytoplasmic organelles, tight cell junctions) and ECM expression (e.g. collagen type IV and fibronectin) [111]. Two layer (epithelium and stromal) [112] and three layer (epithelium, stromal and endothelium) [46] corneas have already been fabricated *in vitro* with adequate transparency and mechanical stability. Production of an organised, full thickness corneal stromal is the major challenge in corneal bioengineering. Substrates with topographical features imitating corneal stromal architecture [113-116], growth factor supplementation and complex culture media have been extensively assessed [117-120]; however, *in vitro* culture of
corneal keratocytes is notoriously difficult due to their trans-differentiation towards myofibroblast lineage [4, 7, 121]. Nonetheless, construction of a full thickness corneal equivalent remains a challenge, primarily due to the low proliferation [120], slow ECM deposition [122] and trans-differentiation issues towards myofibroblast lineage) of corneal stromal cells [7]. To develop scaffold-free therapeutic strategies, it is imperative to understand advances that have been achieved to date in the quest of the ideal in vitro microenvironment for corneal stromal cell phenotype maintenance and differentiation of other cell types towards corneal stromal lineage.

1.3. Engineering functional in vitro microenvironments using biochemical, biological and biophysical signals

Corneal stromal tissue engineering aspires to develop a transparent and mechanically stable tissue construct that will maintain corneal stromal cells functionality [29]. However, bereft of their optimal tissue context and during in vitro expansion, the quiescent keratocytes lose their phenotype, physiological function and therapeutic potential [7]. To this end, a complex milieu, comprised of biochemical (e.g. media supplements, serum supplementation, oxygen tension), biological (e.g. growth factor supplementation, co-culture systems, conditioned media, genetic modification / cell reprogramming) and biophysical (e.g. surface topography, substrate stiffness, localised density) signals alone or in combination, is recruited with variable degree of efficacy for corneal stromal cell phenotype maintenance in vitro (Table 1.3).
<table>
<thead>
<tr>
<th>Nature of Signal</th>
<th>Approach</th>
<th>Characteristics of corneal stromal cells</th>
<th>References</th>
</tr>
</thead>
</table>
| Biochemical     | Media supplements | Collagen types I, V & VI synthesis ↑  
Native corneal stromal morphology stimulated | [123] |
|                 | Serum supplementation | Prostaglandin D & keratan sulphate synthase ↓, ALDH ↓, Decorin ↑ & fibroblastic appearance ↑ with FBS. PGDS & keratin sulphate synthesis ↑  
Dendritic morphology restored in serum free media | [124] |
|                 | Oxygen tension | Number of apoptotic keratocytes ↓  
Transcription factor HIF1–α ↑ | [125-128] |
| Biological      | Growth factor supplementation | Keratan sulphate ↑  
Dendritic morphology, ALDH & keratocan maintained | [120, 129] |
|                 | Co-culture systems | AQP1, B3GNT7, PTDGS, & ALDH3A1 ↑, Keratocan mRNA ↑ | [130] |
|                 | Conditioned media | Keratocan, lumican & ALDH1A1↑  
Lost the expression of α-SMA | [131] |
<p>|                 | Gene delivery | The mRNA expression for α-SMA &amp; type III collagen ↓ | [132] |
| Biophysical     | Surface topography | Keratocytes alignment ↑, α-SMA expression ↓ | [133-135] |
|                 | Substrate stiffness | Soft substrate (lower young modulus) maintain the cell | [136, 137] |
| Multifactorial | Defined keratinocyte serum-free media / Low calcium media | Keratocan, CD34, ALDH &amp; keratocan promoter- driven enhanced cyan fluorescent protein (ECFP) maintained Smad2, Smad4, TGF-beta1, TGF-beta RII activity ↓ | [138] |
| Media supplements / Serum origin (species) / Growth factor | Media supplements / Serum origin (species) / Growth factor | Cell proliferation ↑ CD34 expression maintained | [139] |
| Growth factor / Compressed collagen scaffold | Growth factor / Compressed collagen scaffold | The cell morphology maintained Cell differentiation &amp; Cell migration ↑ | [140] |
| Co-culture / Hydrogel system | Co-culture / Hydrogel system | The fibroblast to keratocytes phenotype switch ↑ | [141] |
| Growth factor / Conditioned media | Growth factor / Conditioned media | Kerocyte cell proliferation ↑ | [142] |
| Serum free / Spheroid culture | Serum free / Spheroid culture | Morphology, keratocan, ALDH, &amp; CD34 maintained | [143] |
| Substrate composition / | Substrate composition / | α-SMA ↓, light scattering by the tissue construct ↓ | [144] |</p>
<table>
<thead>
<tr>
<th>Substrate topography</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth factor / Cytokines / Collagen gel</td>
<td>Matrix contraction ↓ with IGF-I &amp; IL-1α</td>
<td>[119]</td>
</tr>
<tr>
<td>Nano-fibres / Serum-free media / Insulin</td>
<td>Construct contraction ↓, Thy-1 &amp; α-SMA ↓ Cell orientation ↑, ALDH3 expression ↑</td>
<td>[145]</td>
</tr>
</tbody>
</table>
1.3.1. Media supplements

Culture media contain defined concentrations of various salts, nutrients, pH buffering substances and antibiotics to ensure cell growth in vitro and reproducibility. However, for specific cell types, further additives are required to maintain their function ex vivo. Ascorbic acid is traditionally used to induce collagen (types I, V and VI) synthesis without affecting cellular integrity of human [146] and bovine [147] corneal stromal fibroblasts. Further, studies indicate that the addition of ascorbic acid induces human corneal stromal cells to adopt a physiological morphology and spatial distribution and to deposit collagen fibrils that resemble those of the human cornea in appearance and composition [123, 148]. Ascorbic acid supplementation also stimulates the synthesis of collagen types I, V and VI in keratocyte culture [148] and facilitates the production of thin stromal-layers with similar morphology to the developing mammalian corneal stromal after 5 weeks in culture [123]. Human keratocytes cultured with retinoic acid, a metabolite of vitamin A, showed enhanced proliferation and stratification; reduced mobility; promoted expression of keratocan, lumican and decorin; increased the amounts of collagen type I; and reduced expression of MMP 1, MMP 3 and MMP 9 [149]. In bovine keratocyte culture, insulin stimulated the synthesis of collagen, without affecting lumican and keratocan accumulation. In combination with ascorbic acid, collagen synthesis and lumican and keratocan accumulation was stimulated [150]. However, a similar study demonstrated that bovine keratocytes treated with insulin contained significantly higher levels of DNA as compared to the non-treated samples. They also contained higher levels of $\alpha 1$(I), $\alpha 2$(I) and $\alpha 1$(V) collagens and keratocan, lumican and decorin than the non-treated samples and samples treated with IGF-1 [118]. Insulin supplementation promoted expression of collagen type IV, lumican and keratocan [151]. Bovine corneal stromal cells treated with 1% FBS and 1% insulin-transferrin-selenium (ITS) induced secretion and alignment of abundant ECM [152]. Further, in the presence of ITS, as opposed to FGF-2 and PDGF-BB, bovine keratocytes were expanded more effectively, maintained their phenotype and proteoglycan degradation was prevented [120]. Human and bovine keratocytes, when cultured in serum-free media supplemented with ITS, maintained their
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morphology and differentiated into fibroblasts when cultured in the presence of serum. Keratocan was detected in early passage bovine fibroblasts, BMP3 and cadherin were expressed in both human and corneal keratocytes and human and bovine corneal keratocytes and fibroblasts expressed vimentin [153]. However, ITS supplementation failed to maintain monkey keratocyte phenotype [138]. Supplementation of rabbit keratocytes with Acacia honey showed no morphological changes, increased proliferative capacity, increased expression of ADLH and vimentin and no chromosomal changes [154]. Edible bird nest has also been shown to favour rabbit keratocyte growth in vitro, possibly due to the presence of avian epidermal growth factor [155]. However, whether such exotic supplements would reach clinic is questionable.

1.3.2. Serum supplementation

Animal serum supplementation is the most indispensable element of standard cell culture. However, its use the recent years is controversial due to batch-to-batch variability [156] and the potential of host immune reactions [157]. When bovine keratocytes were cultured under serum-free conditions, they synthesised decorin, keratocan, lumican and mimecan and maintained a physiological phenotype [158]. Further, bovine keratocytes in serum-free media were quiescent, appeared dendritic and synthesised high amounts of keratan sulphate, whilst in the presence of 10% FBS, they had high proliferation rate, appeared fibroblastic in morphology and synthesised low amounts of keratan sulphate [25]. Similarly, when bovine keratocytes cultured in serum-free medium appeared dendritic, whilst they became fibroblastic in appearance when exposed to FBS. Both keratocytes and fibroblasts synthesised similar amounts of collagen type I and type V. However, corneal fibroblasts were negative for ALDH and synthesised significantly higher levels of decorin and significantly lower levels of prostaglandin D synthase and keratan sulphate. Of significant importance is that subsequent serum deprivation, although did not restore ALDH to keratocyte levels, did restore cell morphology to a more dendritic appearance and returned the synthesis of decorin, prostaglandin D synthase and keratan sulphate to keratocyte levels [124]. Further, bovine keratocytes keratan
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sulphate PGs expression was decreased during the initial 2 – 4 days of culture in quiescent cultures with low serum concentrations (0.1%). Addition of FBS stimulated cell division but did not stimulate keratan sulphate PGs secretion. Keratan sulphate PG secretion was lost during serial subculture [129]. Rabbit keratocytes cultured under serum-free conditions expressed collagen type IV. However, collagen type IV was not detectable in keratocytes cultured in media supplemented with FBS. Lumican and keratocan were expressed and secreted in serum-free media and were downregulated by FBS [151]. Similarly, equine corneal fibroblasts, keratocytes and myofibroblasts grown under serum-free conditions had a similar morphological appearance as keratocytes in situ. On the other hand cells grown in 10% FBS demonstrated spindle shaped fibroblast morphology. Equine stromal cells cultured in serum-free medium containing TGF-β1 exhibited corneal myofibroblast phenotype. Keratocytes had the lowest proliferation rate, whilst corneal fibroblasts had the highest proliferation rate. Keratocytes were not positive for α-SMA; fibroblasts demonstrated sparse α-SMA expression and myofibroblasts revealed extremely high α-SMA expression [159]. SV40-transformed human corneal keratocytes have also been shown to transform into activated corneal fibroblast phenotypes in response to serum and to maintain quiescent keratocyte phenotype under serum-starved conditions [160], although early studies demonstrated that when human corneal cells were cultured at 5% FBS, they maintained the keratocyte morphology, spatial organisation and ECM deposition [161]. Further, new-born calf serum has been shown to have a greater effect on keratocytes growth than FBS [139]. All in all, data to date indicates that independent of the species, the presence of serum induces differentiation towards fibroblastic lineage, whilst serum-free conditions maintain corneal keratocyte phenotype.

1.3.3. Hypoxia

Oxygen levels in most mammalian tissues are in the region of 2 to 9%, whilst traditional cell cultures are performed at a hyperoxic environment (21% O₂), which has a strong impact on cellular biology [162]. Recent data suggest that hypoxia, or rather physiological low O₂ tension is beneficial for cell phenotype maintenance in
vitro and development of tissue engineered constructs [163]. In corneal keratocyte culture, it has been shown that low oxygen tension protects cells from stress induced death [128]. It has also been suggested that hypoxia reduces TGF-β1 induction of primary rabbit corneal keratocyte to myofibroblast phenotype [164], although previous studies with dermal fibroblasts have demonstrated that hypoxia increases mRNA levels of TGF-β1 [165]. Further, hypoxia has been shown to protect bovine keratocytes from UV stress and cytokine mediated apoptosis [127]. Hypoxia upregulates the activity of prolyl hydroxylases involved in the stabilisation of hypoxia-inducible transcription factor (HIF) [166, 167]. Thus, in fibroblast cultures, hypoxia has been shown to increase mRNA levels of procollagen α1(I) [168], enhancing that way ECM production. For these reasons, hypoxia, or rather physiological O₂ tension, may be beneficial in the accelerated development of cellular constructs, whilst maintaining cell phenotype.

1.3.4. Growth factor supplementation
Various growth factors are expressed in normal and diseased cornea, promote cellular proliferation and migration of corneal cells and play an important role in maintaining corneal transparency [169]. The low proliferative capacity and the transdifferentiation issues in the presence of serum of corneal keratocytes have stimulated intensive investigation of the influence of growth factors in corneal stromal cell culture, with variable results to date. The addition of exogenous IGF-1 generated a cell network structure in human corneal fibroblasts [170]. Further, bovine keratocytes treated with IGF-1 contained significantly higher levels of DNA and keratocan, lumican and decorin as compared to the non-treated samples. IGF-1 treated media contained substantially higher amounts of α1(I), α2(I) and α1(V) collagens than the control cultures [118, 171]. IGF-I and IL-1α increased cell proliferation, but did not alter keratocyte phenotype of rabbit keratocytes [119]. In bovine keratocyte culture, IGF-I stimulated the lowest level of proliferation and the highest levels of collagen synthesis. PDGF and TGF-β1 had intermediate effects on proliferation and collagen synthesis. FGF-2 stimulated the highest level of proliferation, the lowest level of GAG synthesis and inhibited the synthesis of
collagen type I and type III [172]. Further, FGF-2 had the least effect on ECM synthesis under agarose overlay conditions in corneal bovine keratocytes [122, 172]. The FGF-2 used in corneal fibroblast culture may also reduce myofibroblast differentiation during in vitro culture. For example, FGF-2 supplementation in bovine keratocytes stimulated cell division and keratan sulphate PGs secretion. Further, FGF-2 prevented serum-induced keratan sulphate PGs down-regulation. Keratan sulphate PG secretion was lost during serial subculture with or without FGF-2. Expression of keratan sulphate PGs core proteins (lumican, mimecan, and keratocan) was stimulated by FGF-2 [129].

The corneal myofibroblasts grown in the presence of FGF-1 or FGF-2 / heparin had decreased expression of α-SMA, TGF-β receptors and cadherins, decreasing that way myofibroblast phenotype and promoting fibroblast phenotype, suggesting that corneal myofibroblasts and fibroblasts are not terminally differentiated cell types [173]. FGF-2 decreased CTGF mRNA in rabbit corneal fibroblast culture [174]. FGF-2 induced fibroblast differentiation with focal adhesion, fibronectin assembly in culture of rabbit keratocytes [119]. Additionally, FGF-2 / heparin sulphate promoted the downregulation of intracellular keratocan, but not lumican mRNA levels and collagen type IV was not detected in rabbit keratocytes [151]. Further, FGF-2 supported the development of multi-layered stromal tissue within 12 weeks and free of α-SMA fibres, however the cells gradually start losing their CD34 phenotype marker [175]. In addition, although keratocyte morphology was lost, FGF-2 stimulated proliferation, maintained ALDH and keratocan expression and prevented PG degradation under serum-free conditions [120]. PDGF induced fibroblast differentiation with focal adhesion, fibronectin assembly in culture of rabbit keratocytes [119]. Further, PDGF-BB stimulated proliferation, maintained ALDH and keratocan expression and prevented PG degradation in keratocyte culture under serum-free conditions; however their keratocyte morphology was lost [120]. TGF-β2 stimulated expression of type I collagen in human corneal fibroblast culture and potently stimulated α-SMA expression [171]. Similarly, TGF-β supplementation induced human corneal keratocytes and immortalised human corneal fibroblasts spreading, assembly of actin filaments, formation of focal adhesions, and expression
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of α-SMA, which was blocked by the addition of RGD-containing peptides [176]. SV40-transformed human corneal keratocytes have also been shown to transform into activated corneal fibroblast phenotypes in response to TGF-β1 [160]. Lumican and keratocan were downregulated and collagen type IV was not detectable in rabbit keratocytes cultured in media supplemented with TGF-β1 [151]. TGF-β1 induced myofibroblast differentiation of rabbit keratocytes with prominent focal adhesion and fibronectin assembly and expression of α-SMA [119]. Similarly, addition of TGF-β or PDGF in bovine keratocytes stimulated cell division, but did not stimulate keratan sulphate PGs secretion [129]. TGF-β treatment induced both CTGF mRNA and protein in rabbit corneal fibroblasts. CTGF supplementation did not increase α-SMA actin mRNA and protein [174]. TGF-β stimulated expression of CTGF in human corneal fibroblasts and both TGF-β and CTGF increased collagen synthesis in corneal fibroblasts, suggesting that CTGF promotes corneal scarring [177]. EGF increased proliferation of human stromal cells [142] and stimulated differentiation of rabbit corneal keratocytes into a myofibroblast phenotype, with loss of dendritic shape and increased expression of α-SMA [178]. However, when mice keratocytes were cultured under serum-free conditions, supplemented with EGF, FGF-2, B27 supplement and leukaemia inhibitory factor and as spheroids, they maintained their phenotype (e.g. expression of vimentin, keratocan, CD34 and lumican) for more than 12 passages [143]. Such complex media are unlikely to reach clinical setting, whilst it is evidenced that IGF-1 and FGF-2 have beneficial effects in culture of corneal stromal cells, even in serum-free media.

1.3.5. Co-culture systems

Cells, in vivo, are in direct contact with other cells and complex ECM milieu. Thus, co-culture systems that closely imitate the in vivo microenvironment of a specific cell population are under investigation. Data to date indicate that corneal epithelial-stromal co-culture systems maintain keratocytes’ phenotype and promote the reversal of fibroblasts phenotype to keratocyte in encapsulated 3D hydrogels [141]. One more study demonstrates restoration of the quiescent keratocyte phenotype of corneal fibroblasts in the presence of epithelium [116]. Another study assessed
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corneal fibroblasts / corneal epithelial cells; corneal fibroblasts / skin epithelial cells; skin fibroblasts / corneal epithelial cells; and skin fibroblasts / skin epithelial cells co-culture systems. Among them, only the corneal derived cell combination produced a physiologically relevant corneal substitute [112]. Co-culture systems have also been used successfully to trans-differentiate human adipose-derived stem cells towards keratocyte lineage; the derived cells had dendritic morphology with high expression of ALDH3A1 and keratocan [179]. Human embryonic stem cells have also been trans-differentiated towards keratocyte lineage, as evidenced by increased keratocan and PG expression [130]. Further, co-culture of stromal cells with healthy epithelium and endothelium maintained physiological PG synthesis of the stromal cells [180]. Although these preliminary data clearly demonstrate the potential of co-culture systems, more detailed in vitro and in vivo studies are necessary.

1.3.6. Conditioned media

Conditioned medium is rich mediator of cytokines, growth factors and other potent biological molecules that can modulate the behaviour of the cultured cells. For example, epithelial cell conditioned medium inhibited and keratocyte conditioned medium stimulated the proliferation of epithelial cells and keratocytes. The addition of exogenous growth factors was unable to overcome the inhibitory potential of epithelial conditioned medium [142]. Further, bone marrow mesenchymal stem cells were differentiated towards keratocyte lineage in the presence of keratocyte conditioned medium, as evidenced by increased keratocan and ALDH1A1 expression and decreased α-SMA expression [131]. These preliminary results demonstrate the potential of conditioning media in keratocyte culture; nonetheless, the limited data available prohibits drawing of safe conclusions.

1.3.7. Genetic modification / cell reprogramming

Gene delivery, based on viral and polymeric systems, for the treatment of corneal diseases is still under developing stage. The corneal tissue is ideal candidate for gene delivery, due to its ease of access and immune-privilege nature [181, 182]. A single
intra-stromal injection of recombinant AAV–MMP14 gene resulted in reduction in mRNA expression for α-SMA and type III collagen (fibrotic markers) in murine stromal keratocytes *in vitro* and *in vivo* mouse cornea model demonstrated a good therapeutic potential for corneal fibrosis [132]. Decorin transfection of corneal fibroblasts and keratocytes demonstrated reduction in TGF-β induced α-SMA expression and also reduction in various pro-fibrogenic genes, such as fibronectin, collagen type I, type III and type IV [183]. Given the readiness of stromal cells to lose their phenotype during *in vitro* culture and their limited availability, genetically modified corneal stromal cells (SV-40 immortalised human corneal keratocytes) have been engineered due to their consistent growth, lack of α-SMA expression and phenotype maintenance, even in serum-free media [47, 160, 184]. A significant advancement in the field is the development of a full thickness human corneal tissue using engineered cells on a collagen scaffold, which was fully functional and morphologically equivalent to the native tissue [44], however no follow up in *in vivo* models was recorded.

The reprogramming of human corneal keratocytes into iPSCs demonstrates that these cells remained stable for over 30 passages and showed embryonic stem cell-like pluripotent properties. The application of these cells on surgical abrasion-injured cornea demonstrated that these keratocyte-reprogrammed iPSCs enhance the corneal wound healing, whilst in severe corneal damage induced by alkali, these cells with a delivery hydrogel system enhanced corneal reconstruction by downregulating oxidative stress and recruiting endogenous epithelial cells to restore corneal epithelial thickness [185].

**1.3.8. Substrate topography**

Anisotropic electro-spun fibres and imprinted substrates have been used extensively to control the phenotype of corneal stromal cells. Aligned collagen electro-spun fibres (50-300nm diameter) have been shown to downregulate α-SMA and to upregulate corneal fibroblast markers [133, 144], and aligned polyglycolic acid electro-spun fibres have been shown to induce optimum transparency of corneal tissue 8-week post-implantation [186]. Of significant importance is a recent study
that demonstrated aligned electro-spun polylactic acid fibres under serum-free conditions to revert keratocyte phenotype from fibroblast phenotype within 7 days of culture, whilst no cellular contraction was observed [145]. Aligned polyester urethane urea electro-spun fibres with average diameter of approximately 165nm induced alignment of cultured human corneal stromal stem cells with the presence of uniform in diameter collagen fibrils, regular inter-fibrillar spacing and keratocyte phenotype, as evidenced by ALDH and keratocan expression [29]. The combination of growth factors (FGF-2 and TGF-β3) with such aligned nano-fibres further mimicked the human corneal stromal tissue, as evidenced by deposition of multilayered lamellae with orthogonally oriented collagen fibrils [87]. Given the inability to control the spatial distribution of electro-spun fibres, imprinted substrates have been proposed as alternatives. Such anisotropic structures have produced an organised stromal tissue in vitro [187], with physiological cell morphology and gene expression [136, 188, 189]. Further, collagen type I and type III coated silicon surfaces reduced TGF-β induced myofibroblast differentiation and α-SMA expression at size greater than 1400nm [135]. Micro-imprinted poly-ε-caprolactone substrates with dimension 12.5 μm width × 12.5 μm spacing × 4.0 μm depth promoted human keratocyte growth up to 3 weeks and influence cell adhesion (integrins) and tissue remodelling (matrix metalloproteinase) genes [190]. Anisotropic collagen films have also been shown to align corneal keratocytes parallel to the substrate topography and increase expression of collagen type I and keratan sulphate [191]. Further, topographical cues in the nano-range have been shown to upregulate the expression of genes marking differentiated keratocytes (KERA, CHST6, AQP1, B3GNT7) in keratocytes and stromal stem cells culture and to promote organisation of aligned collagen fibrils [192]. An alternative strategy based on magnetic fields has been shown to induce bidirectional orientation of corneal stromal cells [133] and has been used to develop a hemi-cornea (two cells; stromal and epithelium layers) that promoted tissue regeneration in a rabbit model [98].
1.3.9. Substrate stiffness

Tissue culture polystyrene is the most widely used cell supporting material for *in vitro* cell expansion. However, the stiffness of this material is far from physiological stiffness, often resulting in trans-differentiation of cultured cells [193], thus the influence of substrate elasticity is at the forefront of scientific and technological research and development. There are several studies suggesting the active role of substrate’s mechanical property on corneal culture. For example, substrates with stiffness of 40kPa and 130kPa maintained the phenotype of corneal keratinocytes, whilst substrates with stiffness in the region of 1.74MPa caused trans-differentiation within 3 days in culture [194]. In a similar way, surface stiffness also affects the keratocyte phenotype. Indeed, rigid substrates or compressed collagen matrices promoted the contractile fibroblastic phenotype, whilst quiescent phenotype was observed in soft collagen matrices. Further, the TGF-β mediated myofibroblast phenotype was enhanced in the presence of high substrate stiffness [137]. Further, significant higher cellular elongation and change in mRNA expression was observed on hard poly dimethyl siloxane substrates, whilst cells adopted a round morphology on soft chitosan films [136]. Keratocytes completely elongated and adopt spindle-shape morphology and finally lost their native phenotype on mechanically strong collagen substrates [195]. Similarly, TGF-β induced α-SMA myofibroblast trans-differentiation of both human and rabbit keratocytes was inhibited on soft substrates [196].

1.3.10. Three-dimensional / spheroid culture

It has been hypothesised that three-dimensional culture, induced by various scaffold conformations or spheroid culture, would most closely imitate the *in vivo* setting of corneal stromal cells, maintaining their phenotype in culture. Three-dimensional culture of keratocytes on a collagen substrate for 11 weeks resulted in multi-layered tissue-like structure, with cells present in between the alternating parallel and perpendicular arrays of collagen fibrils [197]. Further, corneal keratocytes can be expanded extensively within a three-dimensional compressed collagen matrix, after PDGF induction [140]. Further, collagen sponges have been shown to increase cell
number and promoted continuous secretion of collagen- and PG- rich ECM [198]. Further, incorporation of small bioactive peptide motifs Fmoc-Arg-Gly-Asp-Ser within a collagen hydrogel enhanced human corneal stromal fibroblasts cell adhesion and proliferation and reduced $\alpha$-SMA positive cells [199]. Using a PEG hydrogel, corneal keratocytes remained viable for 4 weeks, exhibited spherical morphologies and maintained keratocan, collagen type I and collagen type III expression, suggesting that three-dimensional cultures can stabilise, but not restore keratocyte phenotype [113]. Rabbit keratocytes remained stable, with reduced $\alpha$-SMA expression and increased ALDH-A1 expression, when cultured using a three-dimensional collagen sponge, as compared to cells grown on two-dimensional collagen films [200]. Three-dimensional collagen scaffolds loaded with IGF and PDGF BB stimulated elongated / dendritic keratocyte morphology, without inducing formation of stress fibres [137]. Further, three-dimensional silk scaffolds promoted human and rabbit corneal fibroblast proliferation, alignment and corneal ECM expression [201].

The first in vitro full thickness cornea equivalent was reconstructed within a three-dimensional collagen gel matrix [42]. Collagen–chondroitin sulphate foam also has been used to develop a human corneal equivalent using primary epithelium and keratocytes and immortalised endothelium [175]. A compressed collagen scaffold has also shown promising results in a corneal stroma of rabbit model [92]. A TERP5 mixed with collagen type I and chondroitin 6-sulphate showed limited activation of $\alpha$-SMA positive myofibroblast-like cells, resulted in excellent epithelial repair and nerve in-growth, restored the basement membrane between the implant and overlying epithelium and completed integrated into the host tissue of Yucatan pigs [202]. Collagen–phosphorylcholine hydrogels showed very good mechanical properties and optical clarity, whilst promoted stromal and epithelium tissue regeneration after 8-12 month implantation [203, 204]. A human recombinant collagen cross-linked with N-cyclohexyl-N-(2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate (CMC) resulted in epithelial and endothelial cell proliferation, neurite outgrowth and clear transparent corneal window within 7 days of transplantation [205]. A human recombinant type III collagen cross-linked with
EDC-NHS was well integrated after 6 months of transplantation and resulted in epithelium, stromal and nerves regeneration [206]. These implants have been shown to remain stable even after 4 years of transplantation, without any sign of rejection [207].

Further, three-dimensional spheroid culture of mouse keratocytes has been shown to maintain their phenotype for more than 12 passages, as evidenced by physiological dendritic morphology and expression of keratocan, ALDH3A1 and CD34 in the presence of serum-free media [143]. Human and bovine fibroblasts converted back to keratocyte-like phenotype after spheroid culture [153]. Of significant importance is that bovine keratocytes cultured in spheroid form remained viable for at least four weeks, secreted more keratan sulphate and keratocan than cells on tissue culture plastic, accumulated substantially more ECM (e.g. keratan sulphate, lumican, keratocan, collagen type V and collagen type VI) in intercellular spaces than cells cultured on tissue culture plastic and both TGF-β and PDGF had minimum effect in cells cultured in spheroid form [208]. Spheroids culture of bovine corneal cells resulted in dendritic cell morphology and expressed vimentin, keratocan, CD34, lumican and ALDH [209]. Further, spheroid culture of corneal stromal cells on attachment-free surface resulted in significantly high expression of keratocan and ALDH3A1 as compared to adherent cells, whereas the expression of α-SMA reduced, closer to the tissue resident keratocytes [210]. In addition, corneal stromal cells derived from BrdU-positive sphere culture were keratocan-positive, had secondary sphere formation capacity and transformed to fibroblast lineage, when grown in two-dimensional culture. Further, these spheroid cultured cells promoted transparency in vivo within four weeks, when implanted to rabbit cornea with a gelatin hydrogel [211]. Thus, spheroids culture is a potential strategy to increase the keratocytes population and also maintaining the cell phenotype.

1.3.11. Localised density

Localised density, in the form of culture overlay, has also been investigated as a means to maintain corneal stromal cells phenotype. Agarose overlay, for example, when used in bovine corneal keratocyte culture, significantly enhanced the cell
number in the IGF-1, TGF-β and PDGF treated cells by 2–3 fold. The overlay also significantly enhanced procollagen conversion to collagen by 63–68% in IGF-1 and PDGF cultures, and by 66–85% in TGF-β cultures [122]. An alternative, yet under-investigated, strategy is based on the principles of macromolecular crowding. Macromolecular crowding (MMC) is a biophysical phenomenon that affects reaction rates and reaction equilibria and plays an important role in cell biology and biophysics, given that the body is made up of several macromolecules in soluble form and/or as structural arrays with total concentrations up to several hundred grams per litre, even in dilute body fluids (80 gram per litre for blood and 36–50 gram per litre for urine). However, the total concentration of solids in culture media is far too low (16.78 grams per litre DMEM / F12, ATCC; 17.22 grams per litre DMEM high glucose and L-glutamine, Invitrogen) [212-214]. MMC is defined as the unavailability of intracellular or extracellular space due to occupation of the available space by macromolecules. The negative charge of the macromolecules increases the volume occupancy due to increased hydrodynamic radii. Thus, the remaining volume (excluded volume) in the system is even less, for other molecules in the system (Figure 1.3) [215]. It has been demonstrated that MMC, through the addition of inert macromolecules in in vitro setting, considerably affects a broad range of biochemical, biophysical and physiological processes, including nucleic acid and protein conformation and stability, protein and DNA interaction equilibrium kinetics, DNA organisation, protein assembly, protein crystallisation, catalytic activity of enzymes, cell volume regulation following the principles of excluding volume effect (EVE) [212, 216-223]. To date, various crowding molecules have been used for skin fibroblasts [224], lung fibroblasts [225, 226], tenocytes [224], osteoblasts [224], bone marrow stem cells [227] and embryonic stem cells [228] and the aforementioned studies have demonstrated accelerated ECM deposition and cell phenotype maintenance (Table 1.4). These studies and various other important studies using MMC principal are also summarised in Table 1.4.
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Figure 1.3. | Macromolecular crowding and excluded volume effect. Originally, the macromolecules have very low volume (A). Once dispersed in media the macromolecules occupy most of the available space, thus, the excluded volume (free space) is very low in this condition (B). The negative charge present on the macromolecules surface (shown in light blue) further reduces the free available volume due to increased hydrodynamic radii of the macromolecules.
Table 1.4. | Examples of macromolecular crowders used and *in vitro* consequence [increased (↑) / decreased (↓) response] in various studies.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Macromolecular crowder used</th>
<th>Purpose</th>
<th>Cell used</th>
<th>Consequence</th>
<th>Year/References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ribonuclease A, beta lactoglobulin, BSA &amp; PEG 20kDa</td>
<td>Effect of MMC upon the structure &amp; function of GAPD</td>
<td>NA</td>
<td>Formation of tetramer ↑</td>
<td>1981 [229]</td>
</tr>
<tr>
<td>2</td>
<td>PEG 8kDa, dextran T-70, Ficoll® 70kDa, BSA</td>
<td>To evaluate the role of DNA polymerase binding to DNA</td>
<td><em>Escherichia coli</em></td>
<td>Non specific enzyme interaction↑ The enzymatic activity ↑</td>
<td>1987 [218]</td>
</tr>
<tr>
<td>3</td>
<td>Dextran Sulphate 500kDa</td>
<td>Effect on pro-collagen processing</td>
<td>Human skin fibroblasts</td>
<td>Complete proteolytic processing of procollagen to collagen</td>
<td>1990 [230]</td>
</tr>
<tr>
<td>4</td>
<td>Dextran 40kDa</td>
<td>Assessment on ECM deposition</td>
<td>Human skin fibroblasts</td>
<td>Deposition of collagen type III ↑</td>
<td>1991 [231]</td>
</tr>
<tr>
<td>5</td>
<td>Dextran Sulphate</td>
<td>To characterise the biosynthesis &amp; matrix deposition in Osteogenesis Imperfecta</td>
<td>Fibroblasts culture</td>
<td>Insoluble matrix deposition ↑ Relative amount of fibronectin ↑</td>
<td>1993 [232]</td>
</tr>
<tr>
<td>6</td>
<td>Ficoll® 70kDa, dextran 70kDa</td>
<td>To assess the chaperonin mediated protein folding</td>
<td><em>Escherichia coli</em></td>
<td>Complete chaperonin folding in the presence of excluded volume</td>
<td>1997 [233]</td>
</tr>
<tr>
<td>7</td>
<td>PEG 6kDa &amp; 35kDa</td>
<td>Effect of total enzyme concentrations on flux through phosphotransferase system (PTS)</td>
<td>Escherichia coli strain (PJ4004)</td>
<td>Enzyme complex dissociation rate constant ↓, Thus the enzyme complex stability ↑</td>
<td>1998 [234]</td>
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</tr>
<tr>
<td>8</td>
<td>PEG 10kDa</td>
<td>To understand the conformational behaviour of a giant duplex-DNA chain</td>
<td>NA</td>
<td>Enhancement of unimolecular condensation of large linear DNA by PEG, The discrete folding transition of the DNA ↑</td>
<td>1999 [235]</td>
</tr>
<tr>
<td>9</td>
<td>BSA, chicken albumin, Ficoll® 70kDa, dextran T70</td>
<td>To study the effect on protein folding &amp; aggregation</td>
<td>Micrococcus lysodeikticus cells</td>
<td>Protein refolding rates ↑</td>
<td>1999 [220]</td>
</tr>
<tr>
<td>10</td>
<td>PEG 300Da</td>
<td>Protein structure &amp; protein stability</td>
<td>NA</td>
<td>↑ in the hydrophobic interaction in the presence of excluded volume influence the folding equilibrium</td>
<td>2001 [236]</td>
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<tr>
<td>11</td>
<td>Dextran (138kDa), PEG (200, 400, 600 &amp; 3350 Da), Ficoll® cocktail (70kDa &amp; 400kDa)</td>
<td>Effect on α-synuclein fibrillation</td>
<td>NA</td>
<td>α-synuclein fibrillation (6-fold with lysozyme; 5-fold with BSA)</td>
<td>2002 [237]</td>
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<td>12</td>
<td>Dextran (0-300 g/l)</td>
<td>Effect on lysozyme protein</td>
<td>NA</td>
<td>Formation of stable, compact, salt-</td>
<td>2003 [238]</td>
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<tr>
<td>13</td>
<td>Dextran sulphate (500kDa) &amp; neutral dextran (670 kDa)</td>
<td>To evaluate the deposition of collagen matrix</td>
<td>WI 38 lung fibroblasts</td>
<td>Collagen deposition ↑</td>
<td>2007 [225]</td>
</tr>
<tr>
<td>14</td>
<td>Ficoll® 70kDa &amp; Ficoll® 70 kDa / 400 kDa</td>
<td>Influence on PCR performance</td>
<td>WI 38 cell derived RNA samples to check the PCR efficiency</td>
<td>Sensitivity (8-10 fold), polymerase processing ↑, Amplicon yield, Primer annealing ↑, specificity ↑, DNA polymerase thermal stability ↑</td>
<td>2007 [239]</td>
</tr>
<tr>
<td>15</td>
<td>Dextran sulphate (500kDa, 10kDa), Polysodium 4-Styrene sulfonate (200kDa), Ficoll® (400 kDa &amp; 70kDa)</td>
<td>To evaluate the deposition of collagen matrix</td>
<td>WI 38</td>
<td>23-fold &amp; 36-fold collagen deposition ↑ after DxS (500 kDa) &amp; PSS (200 kDa)</td>
<td>2007 [226]</td>
</tr>
<tr>
<td>No.</td>
<td>Material/Model</td>
<td>Purpose</td>
<td>Expected Outcome</td>
<td>Year</td>
<td>Reference</td>
</tr>
<tr>
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<tr>
<td>16</td>
<td>PEG-3.5kDa &amp; Ficoll® 70 kDa</td>
<td>To evaluate the effect of crowing on fibrillation of proteins specific to conformation &amp; aggregation behaviour of fibrillating proteins</td>
<td>Fibrillation of oligomeric proteins ↓</td>
<td>2008</td>
<td>[222]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fibrillation of monomeric natively unfolded Proteins ↑</td>
<td></td>
<td></td>
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<tr>
<td>17</td>
<td>PEG 8kDa</td>
<td>To evaluate the encapsulation effect in Giant Lipid Vesicles</td>
<td>Encapsulation efficiency of poorly encapsulated macromolecules in fluorescently labelled polymers ↑</td>
<td>2008</td>
<td>[240]</td>
</tr>
<tr>
<td>18</td>
<td>Ficoll® 70 kDa</td>
<td>To evaluate the influence of MMC on shape &amp; proteins folding dynamics</td>
<td>Borrelia burgdorferi VlsE model</td>
<td>Distinct conformational changes in VlsE proteins are accompanied by secondary structure changes</td>
<td>2008</td>
</tr>
<tr>
<td>19</td>
<td>Dextran</td>
<td>Effect on the structure &amp; condensation of DNA molecule confined in nano-channel</td>
<td>NA</td>
<td>DNA molecules progressively elongate &amp; condense into a compact structure</td>
<td>2009</td>
</tr>
<tr>
<td>20</td>
<td>Dextran sulphate (500kDa), Ficoll® (400kDa &amp; 70kDa)</td>
<td>Development of novel in vitro model to assess the effect of antifibrotic agents</td>
<td>WI-38 cells</td>
<td>The in vitro fibrosis model was developed which can be used in a fast, quantitative &amp; non-destructive way</td>
<td>2009</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>Ficoll&lt;sup&gt;®&lt;/sup&gt; 70kDa &amp; dextran 70kDa</td>
<td>To understand the role of a crowded physiological environment in the pathogenesis of neurodegenerative diseases</td>
<td>Escherichia coli Accelerate the nucleation step of fibril formation of human Tau fragment/human prion protein/humanα-synuclein (a significant decrease in the lag time)</td>
<td>2009 [219]</td>
</tr>
<tr>
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</tr>
<tr>
<td>22</td>
<td>Ficoll&lt;sup&gt;®&lt;/sup&gt; (70kDa &amp; 400kDa)</td>
<td>Effect on ECM deposition</td>
<td>NA</td>
<td>10 fold increase in collagen type I deposition in 2D</td>
<td>2010 [244]</td>
</tr>
<tr>
<td>23</td>
<td>Ficoll&lt;sup&gt;®&lt;/sup&gt; (400kDa &amp; 70 kDa)</td>
<td>To evaluate the impact of induced crowding on extra- &amp; intra-cellular protein organisation of human MSCs</td>
<td>ECM derived from MSCs</td>
<td>Supramolecular assembly ↑, alignment of ECM ↑, Alignment of the intracellular actin cytoskeleton ↑, MMC induced deposited matrix promote adhesion, proliferation &amp; migration of MSCs</td>
<td>2012 [245]</td>
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<td>24</td>
<td>Dextran sulphate (500kDa) &amp; Ficoll&lt;sup&gt;®&lt;/sup&gt; (400 kDa &amp; 70 kDa)</td>
<td>Stable pluripotent feeder-free propagation of human embryonic stem cells (hESCs)</td>
<td>WI 38 derived ECM</td>
<td>ECM deposited by fibroblast under MMC build suitable microenvironment for stable hESC propagation</td>
<td>2012 [228]</td>
</tr>
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<td>25</td>
<td>Ficoll&lt;sup&gt;®&lt;/sup&gt; (70kDa &amp; 400kDa)</td>
<td>Effect on ECM deposition (2D Vs 3D)</td>
<td>Pig chondrocytes</td>
<td>Collagen type II deposition ↑</td>
<td>2013 [246]</td>
</tr>
<tr>
<td></td>
<td>Material</td>
<td>Methodology</td>
<td>Cell Types</td>
<td>Observations</td>
<td>Year [Ref]</td>
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<tr>
<td>---</td>
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<td>------------------------------------------------------------------------------</td>
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<tr>
<td>26</td>
<td>Ficoll&lt;sup&gt;®&lt;/sup&gt; 400kDa</td>
<td>To evaluate the rate of collagen I nucleation, fibre growth &amp; proliferation of MSCs</td>
<td>MSCs</td>
<td>Gel pore size, protein permeability, transparency &amp; resistance to enzymatic degradation can be controlled. Mechanical stability of MMC induced gel ↑ Proliferation of MSC culture ↑</td>
<td>2014 [247]</td>
</tr>
<tr>
<td>27</td>
<td>DxS 500kDa, Ficoll&lt;sup&gt;®&lt;/sup&gt; 70kDa &amp; 400kDa, Carrageenan, PSS 200kDa</td>
<td>Influence on ECM deposition</td>
<td>Human skin fibroblasts (WS 1), WI 38, Tenocytes, Osteoblasts</td>
<td>Deposition of various ECM proteins ↑</td>
<td>2014 [224]</td>
</tr>
<tr>
<td>28</td>
<td>Polyvinylpyrrolidone (PVP)</td>
<td>Use of PVP as a novel crowder molecules to assess the cell behaviour</td>
<td>Bone marrow-derived MSCs, Dermal fibroblasts</td>
<td>Cell proliferation ↑, ECM deposition ↑</td>
<td>2014 [248]</td>
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1.4. Project rationale, hypothesis and objectives

Given the beneficial effects to date of MMC in various cell types and the limitations of the various *in vitro* microenvironment modulators, herein we venture to assess the influence of MMC in corneal fibroblast culture. The driving hypothesis is that MMC will enhance corneal stromal cell ECM deposition, whilst maintaining their phenotype in culture. The following specific objectives are foreseen:

1. To assess the influence of neutral macromolecular crowders (e.g. Ficoll®) in human corneal fibroblast culture;
2. To assess the influence of negative charged macromolecular crowders (e.g. dextran sulphate and carrageenan) in human corneal fibroblast culture.
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Assessment of neutral crowders on corneal fibroblasts culture

The contents of this chapter have been published in Scientific Reports as “Macromolecularly crowded in vitro microenvironments accelerate the production of extracellular matrix-rich supramolecular assemblies”. By Pramod Kumar, Abhigyan Satyam, Xingliang Fan, Estelle Collin, Yury Rochev, Brian J. Rodriguez, Alexander Gorelov, Simon Dillon, Lokesh Joshi, Michael Raghunath, Abhay Pandit and Dimitrios I. Zeugolis. 2015: (5), 8729.
2.1. Introduction

Tissue engineering by self-assembly [1, 2], cell-sheet tissue engineering [3], scaffold-free tissue engineering [4] or modular tissue engineering [5, 6] utilise nature’s sophistication to create bottom up supramolecular assemblies. The rationale of using these approaches lay on the fact that cells are the traditional extracellular matrix (ECM) builders and create tissue modules with precision and stoichiometric competence still unmatched by man-made devices. Such technologies have led to the development of cohesive cell-assembled prototypes, held together by cell-cell and cell-ECM junctions, for example skin [7-9], blood vessel [10, 11] and cornea [12] that have demonstrated reparative / regenerative efficacy, even in clinical setting.

Over 30 years have passed since the development of the first tissue equivalent in vitro [7-9]; it is now evidenced that this scientific breakthrough was not followed by a similar magnitude technological advancement. Indeed, we have now recognised that the major culprit in clinical translation and commercialisation of cell-based therapies is the creation of functional in vitro microenvironments that, by imitating features of the tissue from which the cells were extracted, will facilitate cell phenotype, function and therapeutic potential maintenance ex vivo [13]. Thus, bioinspired research efforts recruit pioneered technologies in biophysical cues (e.g. surface topography, substrate elasticity), biochemical beacons (e.g. oxygen tension, ascorbic acid supplementation) and biological signals (e.g. growth factor supplementation, co-culture systems) to maintain permanently differentiated cells phenotype and/or to accurately direct stem cells lineage commitment for the creation of functional tissue facsimiles in vitro [14-17].

Despite the significant advancements that have been achieved to date, the development of an implantable device remains notoriously difficult and prohibitively expensive, as markedly long ex vivo culture times are required (e.g. 196 days for blood vessel) [10] that are associated with loss of native phenotype and cellular senescence [18, 19]. Herein, we hypothesise that macromolecular crowding (MMC), a biophysical phenomenon known to accelerate biological processes by several orders of magnitude [20-22], will enhance protein turnover and amplify the production of ECM-rich supramolecularly assembled tissue equivalents.
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Specifically, in vivo, cells inhabit in the dense intertwined network of the ECM, where the proteinase-specific conversion of procollagen to collagen type I is rapid [23]. In the dilute culture media, the conversion of water soluble procollagen to water insoluble collagen type I is very slow; thus prolonged culture times are required to create an implantable device (Figure 2.1). Thus, the addition of inert macromolecules in the culture media, by imitating the dense extracellular space, will enable the accelerated production of ECM-rich living substitutes (Figure 2.1). To validate our hypothesis, human corneal fibroblasts (HCFs) were used for very first time as exemplary and the influence of a Ficoll® cocktail (FC) as a means of MMC on collagen type I, the most abundant ECM protein of corneal stroma, biosynthesis was assessed.
Figure 2.1. | Schematic representation of how MMC enhances ECM deposition in vitro and its application in the development of ECM-rich cell sheet. Under standard cell culture conditions, the conversion of the water-soluble procollagen to insoluble collagen is very slow as the proteinases are deactivated before they cleave the specific N- and C- propeptides and/or the procollagen is dissolved before its conversion to collagen. After substantially long culture time, the cells will self-crowd the media and collagen is deposited in the cell layer. The addition of inert polydispersed macromolecules (presented as spheres with different diameters) in the culture media results in most effective volume occupancy; in increased relative density of procollagen and proteinases; in cleavage of the propeptides by their respectful proteinases; and finally in accelerated procollagen conversion to collagen and deposition of the former.
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2.2. Materials and methods

2.2.1. Materials

Tissue culture consumables were purchased from Sarstedt (Ireland) and Nunc (Denmark). All other materials, including cell culture media, reagents and macromolecular crowders (Ficoll®70, Ficoll®400) were purchased from Sigma Aldrich (Ireland), unless otherwise stated. Live/Dead® viability / cytotoxicity kit and alamarBlue® cell metabolic activity kit were purchased from BioSource International, Invitrogen (Ireland).

2.2.2. Corneal fibroblast culture

Primary HCFs (Innoprot, Spain) were cultured as per supplier protocol. Briefly, 5,000 cells/cm² were seeded on poly(L-lysine) coated tissue culture flasks maintained at 37 °C with 5 % CO₂ / 95 % air in a humidified incubator. Cells used in all experiments were between 3 and 5 passage.

2.2.3. Macromolecular crowding treatment

HCFs were seeded in 24-well plates at 25,000 cells/cm² density. When we compared low-density and high-density cultures, 25,000 cells/cm² and 50,000 cells/cm² were used, respectively. The next day, the media was replaced with fresh containing FC (Ficoll®70 + Ficoll®400: 37.5 mg/ml + 25 mg/ml) in the presence of 0.0 to 10.0 % NBCS or HS. 100 μM L ascorbic acid phosphate supplement was added in the media during the crowding experiment to enhance ECM synthesis. The influence of MMC was assessed at 2, 4 and 6 days in culture.

2.2.4. Collagen deposition analysis

Media and cell layers were digested with porcine gastric mucosa pepsin (Sigma Aldrich, Ireland) for 2 hours at 37 °C with continuous shaking and subsequent neutralisation with 1 N NaOH. The samples for SDS-PAGE were prepared using appropriate dilution with distilled water and 5X sample buffer. Finally, 15 μl per sample solution per well was loaded on the gel (5 % running gel / 3 % stacking gel) after 5 minutes heating at 95 °C. Electrophoresis was performed in a Mini-
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PROTEAN Tetra Electrophoresis System (Bio-Rad, Ireland) by applying potential difference of 50 V for the initial 30 minutes and then 120 V for the remaining time (approximately 1 hour). The gels were washed gently with ultra pure water and stained using silver stain kit (SilverQuest™, Invitrogen, Ireland) according to the manufacturer’s protocol. Images of the gels were taken after brief washing with water. In order to quantify the cell-produced collagen type I deposition, the relative densities (GeneTools software, Syngene, Ireland) of collagen α1(I) and α2(I) chains were evaluated and compared to the α1(I) and α2(I) chain bands densities of standard collagen type I (Symatese Biomateriaux, France).

2.2.5. Matrix metalloproteinase analysis
The presence of MMPs was evaluated using gelatin zymography. Briefly, at the end of each cell culture time point, media were collected and mixed with non-reducing SDS sample buffer (125 mM Tris-HCl, pH 6.8; 20 % glycerol; 2 % SDS; 0.002 % bromophenol blue) and fractionated by SDS-PAGE using 10 % gels containing 0.1 % gelatin. After electrophoresis, the gels were washed with two incubations in 2.5 % Triton X-100 for 30 minutes. The gels were incubated for 18 hours at 37 °C in a reaction buffer containing 50 mM Tris, pH 7.4; 5 mM CaCl2; 1 µM ZnCl2 to promote recovery of protease activity and then stained with 0.5 % Coomassie G250 brilliant blue for 30 minutes. Images of the gels were taken after de-staining with 30 % ethanol / 10 % acetic acid. These gelatin zymography gels bands were compared for relative expression of MMP-2. Uncultured media with various percentages of NBCS and HS were also used as controls in respective experiments.

2.2.6. Cell morphology analysis
Cell morphology was assessed using phase contrast microscopy (Olympus IX81 inverted microscope, Japan).

2.2.7. Cell metabolic activity and viability assessment
The influence of MMC on cell metabolic activity and viability was assessed using alamarBlue® and Live/Dead® assays, as per manufacturer’s guidelines. Briefly, cell
culture media containing 10% alamarBlue® reagent was added to various samples after removing the culture medium and brief washing with PBS and then incubated for 4 hours to allow for the reduction of resazurin dye by active cells in cellular metabolism assay. Following incubation, 100 µl of medium samples were transferred into a black 96-well plate. Fluorescence of the media was determined using a microplate reader (Varioskan Flash, Thermo Scientific, UK) at excitation and emission 570 nm / 600 nm wavelength. The level of metabolic activity was calculated using % reduction of dye, according to the supplier’s protocol and compared with the respective control samples. The cell viability was determined using Live/Dead® viability kit (Invitrogen, Ireland). The cells were incubated with calcein-AM and ethidium homodimer solution (2 µM calcein-AM and 4 µM EthD-1) in PBS according to manufacturer’s staining protocol for at least 30 minutes. The cell layers were washed in fresh PBS to remove excess dye. Following that, fluorescence images were taken using an Olympus IX81 inverted fluorescence microscope.

2.2.8. DNA quantification
DNA quantification was carried out using Quant-iT™ PicoGreen® dSDNA assay kit (Invitrogen, Ireland) according to the manufacturer’s protocol. Briefly, DNA was extracted using three freeze-thaw cycles after adding 200 µl of nuclease free water per well (24 well plate). The cell suspension was subsequently transferred to cold eppendorf tubes and was centrifuged for 5 minutes at 12000 RPM. 25 µl were then transferred into 96-well plate containing 75 µl of 1xTE buffer. A standard curve was generated using 0, 7.8, 15.6, 31.2, 62.5, 125, 250 and 500 µg/ml DNA concentrations. 100 µl of a 1:200 dilution of Quant-iT™ PicoGreen® reagent was added to each sample and the plate was read using a micro-plate reader (Varioskan Flash, Thermo Scientific, Ireland) with an excitation wavelength of 480 nm and an emission wavelength of 525 nm.

2.2.9. Immunocytochemistry analysis
Cells were seeded in 4-well-chamber Lab-Tek™ II slide and MMC was carried out as described above. At the end of each culture time point, cells were fixed with 3 %
formaldehyde in PBS (pH 7.4). After fixation, the specimens were washed three times in PBS. The fixed cell layers were incubated with 3 % BSA for 30 minutes to stop the non-specific binding of proteins. Cell layers were incubated with primary antibodies diluted in PBS for 90 minutes at room temperature. Rabbit collagen type I, III, IV, V and VI (Abcam, UK; 1:200) and mouse anti-fibronectin antibody (Sigma Aldrich, Ireland; 1:200) were used to detect the expression of various collagens and fibronectin respectively. Mouse anti-actin, α smooth muscle antibody (1:400 dilution), mouse CD34 antibody (1:50 dilution) and mouse keratocan antibody (1:50 dilution) were used to detect α-SMA, CD34 and keratocan expression in HCFs culture. Methanol fixing (cold methanol at -20 °C for 5 minutes) was also used after fixation to detect CD34. After primary incubation, the samples were incubated with secondary antibody for 30 minutes at room temperature. The secondary antibodies used were Alexa Fluor® 488, chicken anti rabbit or donkey anti mouse respectively for rabbit and mouse antibodies at the 1:400 dilutions (Invitrogen, Ireland). Antibody incubation was followed by three washes in PBS. For nuclear staining, DAPI (4’,6-diamidino-2-phenylindole) was used at 1:4000 dilution (Invitrogen, Ireland). Finally, the cover slips were mounted on glass slides with VectaShield (Vector Laboratories, UK) for direct observation. The images were taken using an Olympus IX81 inverted fluorescence microscope using 10X objective. The fluorescence intensity was evaluated using Scope-Pro Plus software.

2.2.10. Cell sheet production

Poly(NIPAAM-co-NTBA) was dissolved in anhydrous ethanol at 40 µg/ml and subjected to continuous shaking overnight. This acrylamide solution was mixed with poly(L-lysine) (100 µg/ml) in 1:1 V/V ratio and again allowed to shake overnight to mix properly. 100 µl of the mixed solution was deposited onto each of the petri dishes, which were left in ethanol soaked desiccator overnight. The dishes were further dried in 600 mBar vacuum oven at 40 °C for at least 4 hours. Cell culture was carried out as described above after mild UV sterilisation of the petri dishes for 2 hours. Following culture, a temperature-controlled plate was used to induce detachment and subsequent cell-sheet harvesting.
2.2.11. Histological analysis using van Gieson staining

After 6 days in culture, samples were fixed with 3 % paraformaldehyde for at least 30 minutes. The samples were then incubated with Weigert working solution for 10 minutes after three brief PBS washings. Finally, the samples were incubated in van Gieson’s solution for 2-3 minutes, after brief PBS washing. The samples were dehydrated using 95% alcohol, absolute alcohol and xylene. Images were taken using the Olympus microscope (BX 51) at 100X.

2.2.12. Histological analysis using Masson's trichrome staining

Samples were fixed in Bouin’s solution for 1 hour at 56 °C after fixation. The samples were then incubated in Weigert's iron haematoxylin staining for 10 minutes, followed by rinsing with running tap water. The samples were then washed with distilled water, after 10 minutes in warm running tap water. The samples were incubated in Biebrich scarlet-acid fuchsin solution for 10 to 15 minutes and washed again with distilled water. Phosphomolybdic-phosphotungstic acid solution was used for further 15 minutes and the samples were then transferred directly to aniline blue solution and stained for 10 minutes, rinsed briefly in distilled water and then in 1 % acetic acid solution for 5 minutes. The samples were dehydrated quickly through 95 % ethyl alcohol, absolute ethyl alcohol (these steps wipe off Biebrich scarlet-acid fuchsin staining) and cleared in xylene after brief washing in distilled water. Images were then taken using the Olympus inverted microscope (BX 51) at X100.

2.2.13. Atomic force microscopy analysis

HCFs were seeded in 4-well-chamber Lab-Tek™ II slides and MMC (FC) was done as described above. After six days in culture, cell layers were washed with HBSS and fixed at room temperature for 15 minutes. The cell layers were then washed three times with PBS and serially dehydrated with 30 %, 50 %, 70 %, 90 % and 100 % ethanol. Atomic force microscopy (MFP-3D, Asylum Research, USA) analysis was then performed using rectangular Si cantilevers (SSS-NCH, Nanosensors, Switzerland), each having a nominal resonance frequency of 330 kHz and a spring
constant of 42 N/m. Images were recorded using amplitude modulation mode in an ambient environment after drying the samples with nitrogen.

2.2.14. Light transmission
Light transmission was assessed using a previously described method [24]. Briefly, the media was aspirated and 100 µl PBS was added in each sample well after PBS washing. PBS alone was used as control blank. For zero absorbance and 100 % absorbance, the wells without PBS or cells and black dye were taken as control; this black dye showed approximately 100 % absorbance (0.0 % transmittance), while the absorbance value in air was taken as 100 % transmittance. The optical density of the samples was measured using a spectrophotometer (Varioskan Flash, Thermo Scientific, Ireland) at 380 - 780 nm wavelength with a resolution of 5 nm. The % transmission was calculated as:

\[
\text{% Transmittance} = 100 \left(1 - 10^{-A}\right)
\]

where A is absorbance measured.

2.2.15. Gene expression (qRT-PCR) analysis
Total RNA was extracted using a modified Trizol isolation method at the given time points. Briefly, TriReagent® (Invitrogen, Ireland) was added to the cell layer after aspiration of medium from the culture and brief washing with PBS. The cellular layer was mechanically disrupted using gentle pipetting of tissue culture plate. Phase separation was conducted with chloroform and the total RNA contained in the aqueous phase was purified using RNeasy® mini kit column (QIAGEN, Hilden, Germany), according to the supplier’s protocol. Three extractions were carried out for each sample and pooled at the end of the RNeasy protocol. The purity and quantity of total RNA were evaluated using an ultraviolet spectrometer (NanoDrop ND-1000 Spectrophotometer, Thermo Scientific, Ireland). Reverse transcription (RT) was performed using MJ Research PTC-200 DNA Engine system according to the manufacturer’s protocol (Promega RT System, UK). The prepared cDNA was monitored using SYBR® Green master mix (QIAGEN, Hilden, Germany) by real-time PCR using StepOnePlus™ Real-Time PCR System (Applied Bioscience,
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Switzerland). Gene transcription was normalised to the transcription of housekeeping human 18S gene. The $2^{-\Delta\Delta C_t}$ method was used to calculate relative gene expression for each target gene at respective time point. Various primers used for the study are given in Table 2.1.

**Table 2.1.** List of primers used for the qRT-PCR of various ECM molecules and HCFs markers.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen Type Iα1</td>
<td>CTGTAAACTCCCTCC ATCCC</td>
<td>GTCCATGTGAAATTGT CTCCC</td>
</tr>
<tr>
<td>Collagen Type IIIα1</td>
<td>CTGGGGAAATGGAGC AAAAC</td>
<td>AAAGCAAAACAGGGCC AAC</td>
</tr>
<tr>
<td>Collagen Type IVα1</td>
<td>ACGACATCATCAAAG GGGAG</td>
<td>ACCCCAATCCTGTA ACAC</td>
</tr>
<tr>
<td>Collagen Type Vα1</td>
<td>ACCACCAAATTCCTC GACC</td>
<td>CTCAAAACACCTCTCT ATCC</td>
</tr>
<tr>
<td>Collagen Type VIα1</td>
<td>ATCGGACCTAAAGGC TACC</td>
<td>TCTCCCCCTTTCACC ATC</td>
</tr>
<tr>
<td>Fibronectin 1 (FN1)</td>
<td>GGACCAGGACCAAC AAAAAAC</td>
<td>AGACACTAACCACAT ACTCCAC</td>
</tr>
<tr>
<td>Actin α2 Smooth Muscle, aorta (ACTA2) - Transcript Variant 1</td>
<td>GCTGTTTTCCCATCC ATTGTG</td>
<td>CCTCTTTTGCTCTTG CTTC</td>
</tr>
<tr>
<td>Cluster Differentiation molecules 34 (CD34) - Transcript Variant 1</td>
<td>ACATCTCTACGCC AACC</td>
<td>CACGTTCACCCAAAG AAGACC</td>
</tr>
<tr>
<td>N-acetylglucosamine-6-O- Sulfotransferase-6 (CHST6)</td>
<td>TTAGGCCAGAAAAGG GGAGGG</td>
<td>TCAGGTGGAGGAAAG CAGAG</td>
</tr>
</tbody>
</table>
2.2.16. Proteomics analysis

Total protein extraction from the cell layers was carried out using Qproteome™ mammalian protein preparation kit (Qiagen, UK). Briefly, cell layers were washed twice with PBS and scraped gently, using a cell scraper, in the presence of ice cold PBS and transferred to pre-chilled 1.5 ml tubes. This solution was centrifuged at 450 g for five minutes at 4 °C. The cell layer was lysed gently with cell lysis buffer containing Benzonase® nuclease and protease inhibitor after discarding the supernatant. This suspension was centrifuged again at 14,000 g at 4 °C for 10 minutes, after agitation in a rotary shaker for 5 minutes. After centrifugation, the supernatant was transferred into pre-chilled 0.5 ml tubes (Protein LoBind Tubes, UK) and freeze-dried. High-throughput proteomic profiling was performed using the 4-plex iTRAQ labelling kit, equipped with the 4700 MALDI TOF/TOF system (Applied Biosystems, USA). Briefly, the protein pellet from each sample was re-suspended in a protein dissolution buffer (10 mM triethyl ammonium bicarbonate, pH 8.5) and each protein sample was assigned to one of four isobaric tags as per the manufacturer’s instructions. The tagged samples were pooled together and subjected to mass spectrometry analysis. Samples were fractionated using strong cation exchange and separated by reverse phase chromatography using the UltimatePlusNanoLC system (Dionex, USA). After reverse phase chromatography, the eluted protein fractions were spotted onto a MALDI plate (ABI 4800 OptiTOF, Applied Biosystems, USA). Using a Probot printing robot (Dionex, USA), the spotted plate was mixed with α-Cyano-4-hydroxycinnamic acid ionisation matrix (Sigma Aldrich, Ireland) at a ratio of 1:2. Mass spectrometry analysis of the spotted plate was carried out an ABI 4800Plus MALDI-TOF/TOF tandem MS system (Applied Biosystems, USA). The time-of-flight of the protein was proportional to the molecular weight. The final read-out graph was a multi-peak spectrum with different peak intensities, which correspond to the relative protein amount. The peak location corresponds to the precise protein or peptide molecular weight. Data analysis was performed using Protein Pilot 2.0 software (Applied Biosystems, USA) on SWISS-PROT, TrEMBL (www.ebi.ac.uk/swissprot) and NCBI (www.ncbi.nlm.nih.gov/) non-redundant protein databases. The unused score was
kept at more than 1.3 to get the confidence interval more than 90; relative peptide value at 95 % was compared among samples. Selected ECM proteins detected in proteomics results were validated using ICC and fluorescent intensity, using Olympus IX81 inverted microscope.

2.2.17. Statistical analysis
All results presented are mean ± SD. Statistical analysis (MINITAB™ version 16, Minitab, Inc., USA) was performed using two-sample t-test for pair wise comparisons or one away analysis of variance (ANOVA) for multiple comparisons after confirming: (a) the normal distribution from which each of the samples (Anderson-Darling normality test); and (b) the variances of the population of the samples were equal to one another (Bartlett’s and Levene’s tests for homogeneity of variance). Non-parametric statistics were used if above assumptions were violated and consequently Kruskal-Wallis test for multiple comparisons or Mann-Whitney test for 2-samples used. Each experiment was performed in biological triplicates in minimum three samples. Differences between selective experimental groups were considered statistically significant at $p$ value < 0.05.
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2.3. Results

2.3.1. Identification of optimal newborn calf serum (NBCS) concentration for maximum ECM deposition

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; Figure 2.2) and complementary densitometric analysis (Figure 2.3) demonstrated that at all NBCS concentrations collagen type I remained in the media in the absence of FC, whilst in the presence of FC, collagen type I was deposited in the cell layer. It was further observed that collagen type I content remained constant or reduced as a function of increased NBCS concentration at given time point in culture. This reduction in collagen type I content was attributed to the enhanced matrix metalloproteinase-2 (MMP-2) activity, as revealed by gelatin zymography (Figure 2.4). The highest \( p < 0.0001 \) collagen type I deposition was achieved after culturing HCFs for 6 days in the presence of FC. No significant difference \( p > 0.05 \) in collagen type I deposition was observed among the different NBCS concentrations (0 to 10 %) at any time point tested (2, 4 and 6 days). Phase contrast microscopy (Figure 2.5) revealed that HCFs maintained their spindle-shaped morphology for all experimental conditions (e.g. presence / absence of FC; 0.0% – 10% NBCS; 2 – 6 days in culture). Further, the addition of FC did not affect cell metabolic activity (Figure 2.6A) and cell viability (Figure 2.6B & Figure 2.6C) independently of the experimental condition.
**Figure 2.2.** SDS-PAGE analysis revealed that in the absence of FC collagen remained in the media and its content was reduced as a function of time in culture and % NBCS. In the presence of FC, collagen I was deposited at the cell layer and its content was increased as a function of time in culture.
Figure 2.3. | Densitometric analysis of SDS-PAGE (Figure 2.2) semi-quantitatively demonstrates the increased collagen deposition in the cell layer in the presence of FC as a function of time in culture. Increase NBCS concentration did not affect collagen deposition.
Figure 2.4. The reduction in collagen content in the media as a function of increased NBCS concentration and time in culture is attributed to the enhanced MMP-2 content, as revealed by gelatin zymography.
Figure 2.5. | Phase contrast microscopy revealed that HCFs maintained spindle-shaped morphology for all time points (2, 4 & 6 days) independently of the presence or absence of FC and NBCS concentration (0 - 10%).
Figure 2.6. HCF metabolic activity and viability as a function of FC presence and NBCS concentration. (A) MMC (FC) did not affect cellular metabolic activity, as revealed by alamarBlue® assay. (B and C) MMC (FC) did not affect cell viability, as revealed by Live/Dead® assay.
2.3.2. Identification of optimal serum origin for maximum ECM deposition

To avoid xenogeneic contaminants, the influence of human serum (HS), as an alternative to NBCS, was assessed at 0.5 %, in presence and absence of FC for 2, 4 and 6 days. SDS-PAGE (Figure 2.7) and complementary densitometric analysis (Figure 2.8A) showed that in the absence of FC collagen type I remained in the media and its content remained constant as a function of time in culture. This indifference in collagen type I content was attributed to the increased MMP-2 content, as revealed by gelatin zymography (Figure 2.8B). In the presence of FC, collagen type I was deposited at the cell layer and its content was increased as a function of time in culture. The highest ($p < 0.0001$) collagen type I deposition was achieved after culturing HCFs for 6 days in the presence of FC. Significant increase in collagen type I ($p < 0.001$) deposition was observed in the presence of HS, as compared to NBCS at 4 and 6 days. The increase in collagen deposition in the presence of HS was attributed to the lower MMP-2 content of HS (Figure 2.9).

Immunocytochemistry (ICC) analysis (Figure 2.8C) further corroborated the high collagen type I deposition in the presence of FC and its relative increase as a function of time in culture, independently of the serum origin. Phase contrast microscopy (Figure 2.10) revealed that HCFs maintained their spindle-shaped morphology for all experimental conditions (e.g. presence / absence of FC; 0.5% NBCS / HS; 2 – 6 days in culture). Further, the presence of FC did not affect cell metabolic activity (Figure 2.11A), cell viability (Figure 2.11B & Figure 2.11C) and DNA content (Figure 2.11D), at any given time point, independently of the serum origin.
Figure 2.7. To avoid xenogeneic contaminants, the influence of human serum (HS), as an alternative to NBCS, was assessed at 0.5 %, in presence and absence of FC for 2, 4 & 6 days. SDS-PAGE analysis showed that in the absence of FC collagen remains in the media and its content is reduced as a function of time in culture. In the presence of FC, collagen I was deposited at the cell layer and its content is increased as a function of time in culture.
Figure 2.8. MMC accelerates ECM deposition in HCFs culture in the presence of NBCS and HS. (A) Densitometric analysis of SDS-PAGE confirmed the high collagen type I deposition in the cell layer as early as 2 days in culture. Collagen type I deposition was consistently increased up to 6 days in culture (time point assessed). (B) Gelatin zymography detected the 68-72 kDa proMMP-2 and the 62 kDa MMP-2 active form. Both forms of enzyme were higher in NBCS, which explains the enhanced ECM deposition in the presence of HS. (C) ICC analysis further collaborated the enhanced collagen type I deposition in the presence of FC.
Figure 2.9. | Gelatin zymography of the medium containing various concentration of human serum or animal serum (0 - 10%) demonstrates that inherent high MMP activity of animal sera.
Figure 2.10. | Phase contrast microscopy revealed that HCFs maintained their spindle-shaped morphology for all time points (2, 4 & 6 days) independently of the presence or absence of FC, NBCS or HS.
Chapter 2

**Figure 2.11.** | HCF metabolic activity, viability and DNA assessment. MMC (FC) did not affect cellular metabolic activity (A), viability (B, C) and DNA content (D) at a given time point, independently of the serum origin, as revealed by alamarBlue® assay, Live/Dead® assay and Quant-iT™ PicoGreen® dSDNA assay, respectively.
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2.3.3. Production and characterisation of ECM-rich HCFs supramolecular assemblies

Due to the abundant ECM deposition in the presence of FC, commercially available temperature-responsive N-isopropylacrylamide (NIPAAM) coating was not suitable for the detachment of intact ECM-rich HCFs supramolecular assemblies after 6 days in culture, although cell attachment and growth was not prohibited (Figure 2.12). A 65 % N-isopropylacrylamide / 35 % N-tert-butylacrylamide (NTBA) copolymer [poly(NIPAAM-co-NTBA)] allowed attachment (Figure 2.13A) and detachment (Figure 2.13B) of intact ECM-rich HCFs supramolecular assemblies after 6 days in culture in the presence of FC and 0.5 % HS. Auxiliary time-lapse microscopy revealed that due to the abundance in deposited ECM under MMC conditions, the detachment rate of the ECM-rich HCFs supramolecular assemblies was slower than their non-MMC counterparts (Figure 2.13C & 2.14).

Histological analysis using van Gieson’s (Figure 2.15A) and Masson’s trichrome (Figure 2.15B) staining and ICC analysis (Figure 2.15C) further corroborated the enhanced collagen type I deposition under MMC conditions (0.5 % HS, FC, 6 days in culture). Transmittance analysis (Figure 2.15D) revealed no significant difference ($p > 0.05$) between the groups (non-MMC, 0.5% HS, 6 days in culture; MMC, 0.5% HS, 6 days in culture; PBS; control surface). Further atomic force microscopy analysis (Figure 2.15E & 2.16) revealed the presence of abundant quartered staggered collagen type I fibrils at the intercellular regions of the MMC HCFs (0.5 % HS, 6 days in culture).

MMC (0.5 % HS, FC) did not affect ($p > 0.05$) gene expression analysis (Figure 2.17) for collagens (I, III, IV, V, VI); fibronectin; phenotypic markers (CD34, CHST6); and unintended differentiation marker ($\alpha$-SMA) for all time points assessed (2, 4 and 6 days). Proteomics analysis revealed that under MMC conditions (0.5 % HS, FC, 6 days), the total protein content in the cell layer was significantly increased (Table 2.1). Subsequent validation using ICC (Figure 2.18) and complementary fluorescence intensity (Figure 2.19) further confirmed the high deposition of collagenous proteins (I, III, IV, V, VI) and fibronectin under MMC conditions (0.5 % HS, FC, 6 days in culture), whilst no difference was observed for unintended
differentiation markers (α-SMA) and phenotypic markers (CD34, keratocan; Figure 2.20) for all time points (2, 4 and 6 days in culture).
Figure 2.12. | Due to the abundance in ECM deposition in the presence of 0.5 % HS and FC, commercially available temperature-responsive NIPAAm-coated dishes, although allowed cell attachment and growth (A & B), did not allow detachment of intact ECM-rich HCFs cell sheets after 6 days in culture (C, D).
Figure 2.13. | A poly(NIPAAM-co-NTBA) temperature-responsive copolymer allowed production and detachment of dense and cohesive corneal stromal layers. 

(A) The poly(NIPAAM-co-NTBA) temperature-responsive copolymer allowed attachment and spreading of HCFs under MMC and non-MMC conditions. (B) The poly(NIPAAM-co-NTBA) temperature-responsive copolymer allowed intact detachment of the de novo produced ECM-rich HCFs sheets after 6 days in culture. (C) The presence of FC, which resulted in enhanced ECM deposition, delayed the intact detachment of the ECM-rich HCFs sheets; nonetheless, complete detachment achieved within 45 minutes after temperature reduction.
Figure 2.14. | Time-lapse microscopy demonstrated slow detachment of HCFs cell sheets treated with MMC (FC) due to the enhanced ECM deposition, after reduction of temperature below to lower critical solution temperature of copolymer.
Figure 2.15. MMC allowed production of HCFs sheets with physiological, tissue-like characteristics. (A & B) Histological analysis using van Gieson’s and Masson’s trichrome staining demonstrate enhanced ECM deposition and tissue-like organisation of the HCFs substitutes under MMC conditions. (C) ICC analysis for collagen type I further corroborates the enhanced collagen type I deposition under MMC conditions. (D) No significant difference in transparency between MMC HCFs sheets and PBS, control surface and non-MMC sheets was observed at 0.5 % HS and after 6 days in culture. (E) AFM analysis at the intracellular regions (shown are amplitude images) further confirmed the high deposition of collagenous proteins exhibiting characteristic D-periodicity in the presence of FC.
Figure 2.16. | AFM analysis of cell-layers produced after 6 days in culture further confirms the presence of high quarter staggered collagenous matrix in the crowded (FC) samples.
Figure 2.17. | qRT-PCR analysis demonstrated that MMC did not affect gene expression profile of HCFs at respective time points. The expression of collagens (I, III, IV, V, VI); glycoproteins (fibronectin); phenotypic markers (CD34, CHST6); and unintended differentiation marker (α-SMA) for all time points assessed (2, 4 and 6 days) remained unaffected under the conditions tested (0.5% HS, with or without FC).
Figure 2.18. MMC enhanced ECM deposition in the cell layer of HCFs. Validation of proteomics analysis with ICC further confirmed the high deposition of collagenous proteins (I, III, IV, V, VI) and glycoproteins (fibronectin) in the presence of FC, whilst no difference was observed for unintended differentiation marker (α-SMA) at all time points (2, 4 and 6 days). A high level of structural alignment is also evidenced.
**Figure 2.19.** Relative immunofluorescence intensity confirmed the high deposition of collagenous proteins (I, III, IV, V, VI) and glycoproteins (fibronectin) under MMC conditions (0.5 % HS, FC), whilst no difference was observed for unintended differentiation marker (α-SMA) at all time points (2, 4 and 6 days).
Figure 2.20. | Immunofluorescence images for CD34 and keratocan after 2, 4 and 6 days in culture, with and without FC, in the presence of 0.5 % HS. Our data indicate that the cells did not express these markers. HCFs cease expression of these markers rapidly in culture and after exposure to serum.
Table 2.2. | Mass spectrometry results of various proteins in HCFs culture in the presence of 0.5 % HS under non-MMC (control) and MMC conditions (FC) after 6 days in culture. PC indicates Peptide Count; ND indicates Not Detected.

Table 2.2A. | Collagen and collagen related proteins in cell layer

<table>
<thead>
<tr>
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<td>E7ENL6_HUMAN</td>
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Table 2.2C. | PGs in cell layer

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Table 2.2D. | Laminin in cell layer

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<td>Q6UYC3</td>
<td>Q6UYC3  _HUMAN</td>
<td>Nucleus</td>
<td>Nuclear assembly, chromatin organisation, nuclear membrane and telomere dynamics</td>
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<td>Filamin A, alpha (Actin binding protein 280) OS=Homo sapiens</td>
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<td>Q5HY54_ HUMAN</td>
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<td>Act as scaffold for a wide range of cytoplasmic signalling protein</td>
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Table 2.2E. | Tubulins in cell layer

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<td>GN=TUBB2B PE=1 SV=1</td>
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<td>Microtubule assembly</td>
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<td>Tubulin, alpha 3, isoform CRA_c OS=Homo sapiens</td>
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<td>G3V1U9</td>
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<td>GN=TUBA1A PE=3 SV=1</td>
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Table 2.2F. | Proteolytic activity in cell layer

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<td>OS=Homo sapiens</td>
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<td>May be involved in actin cytoskeleton re-organisation by cleaving PTK7</td>
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<td>Plasminogen</td>
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<td>Plasmin dissolves the fibrin of blood clots and acts as a proteolytic</td>
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<td>GN=PLG PE=1 SV=2</td>
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<td>factor in a variety of processes: embryonic development, tissue</td>
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<td>Inhibit proteinase activity</td>
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### Table 2.2G. | Other ECM and cytoskeleton proteins

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<td>Regulate the activity of kinases such as PKC and SR</td>
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<td>Cytoskeleton-associated protein 4</td>
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<td>Q07065</td>
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<td>Mediates the anchoring of the endoplasmic reticulum to microtubules</td>
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<td>P61158</td>
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<td>OS=Homo sapiens GN=ACTR3 PE=1 SV=3</td>
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<td>Regulation of actin polymerisation, formation of branched actin networks</td>
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<td>G3V1Y7</td>
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<td>Cytoskeleton</td>
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<td>Muscle contraction, protein binding, actin dependent ATPase activity and motor activity</td>
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<td>Myosin-9 OS=Homo sapiens</td>
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<td>GN=MYH9 PE=1 SV=4</td>
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<td></td>
<td>Cytokinesis, cell shape, and specialized functions such as secretion and capping</td>
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<td>Lysosome-associated membrane glycoprotein 1 OS=Homo sapiens GN=LAMP1 PE=1 SV=3</td>
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<td>H2A 1A_HUMAN</td>
<td>Nucleus</td>
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<td>PRDX 1_HUMAN</td>
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<td>P57053</td>
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<td>P17931</td>
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<tr>
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</tbody>
</table>
2.3.4. Low-density versus high-density culture

At day 6, MMC at low-density (25,000 cells/cm²) culture induced higher ECM deposition than non-crowded high-density (50,000 cells/cm²) culture (Figure 2.21). The highest ECM deposition was detected in the high-density culture in the presence of MMC (Figure 2.21).
Figure 2.21. | MMC is more effective in producing ECM-rich living substitutes than high-density non-crowded cultures. At day 6, MMC at low-density (LD; 25,000 cells/cm$^2$) culture induced higher ECM deposition than non-crowded high-density (HD; 50,000 cells/cm$^2$) culture and the highest ECM deposition was detected in the high-density culture in the presence of MMC, as evidenced by SDS-PAGE (A) and complementary densitometric (B) analysis, immunocytochemistry analysis (C, D, E) and staining assays (F, G).
2.4. Discussion

During development, cellular secretome creates a tissue-specific microenvironment that governs the various functions of the tissue. Cellular secretome also provides necessary signals for tissue repair and regeneration during or following injury. Therapeutic strategies based on the principles of tissue engineering by self-assembly aim to capture this specific reparative potential of cellular secretome. However, for such approaches to succeed, it is essential to create a functional \textit{ex vivo} microenvironment that by emulating the tissue from which the cells were extracted from or are to be implanted will maintain cell phenotype, function and therapeutic potential. Unfortunately, the still primitive culture conditions not only are associated with phenotypic drift, but also require a prolonged period of time for the development of an implantable device. Herein, we hypothesised that MMC, by imitating the dense extracellular space, will accelerate the deposition of tissue-specific ECM, whilst preserving cell phenotype. Given that tissue engineering by self-assembly strategies for corneal stromal require 4 to 8 weeks for the production of supramolecularly assembled monolayers [24-26] and the readiness of corneal fibroblasts to trans-differentiate towards unintended myofibroblast lineage [27-32], we used this cell type as model.

In the absence of MMC, collagen type I remained in the media, whilst in the presence of FC ample ECM deposition was evidenced in the cell layer as early as 48 hours in culture, which persisted up to 6 days in culture (longer time point tested). This is in accordance to previous observations, where FC accelerated ECM deposition in human lung fibroblast culture [33] and human bone marrow stem cell culture [34]. The efficacy of FC to induce accelerated ECM deposition lays on the fact that effectively excludes volume [35, 36], facilitating that way cleavage of C- and distant N-propeptides by their respective proteinases and therefore accelerated conversion of procollagen to collagen type I.

In tissue engineering by self-assembly, supramolecular aggregates are customarily produced using copious amounts of animal sera (up to 30%). Our data indicate that excessive sera supplementation is counter-effective, due to the high amount of MMP-2 present that degrades deposited ECM. Given though that sera contain survival signals, mitogens and growth factors, all necessary for cell expansion, a
balance needs to be achieved that will allow both cell survival and ECM deposition. Herein, we demonstrate that low serum concentration appears to achieve optimal balance between matrix deposition and cell growth, which is in accordance to previous results, where high serum supplementation resulted in phenotypic drift of corneal stromal cells [37-39], tenocytes [40] and retinal progenitor cells [41], whilst low serum or serum-free media resulted in phenotype maintenance of dental-derived stem cells [42], embryonic stem cells [43] and mesenchymal stem cells [44, 45]. Further, given the potential of interspecies transmission of disease and severe immune reactions associated with animal sera, our data further corroborate previous studies supporting the use of HS for clinically relevant cell therapies [46-50].

The ample deposition of ECM in the presence of FC prohibited harvesting of intact supramolecularly assembled ECM-rich HCFs substitutes from the brush-like commercially available NIPAAM dishes. The efficiency of poly(NIPAAM-co-NTBA) copolymer to produce intact ECM-rich cell sheets is attributed to the decreased number of N – H residues, due to the additional steric hindrance induced by the addition of the NTBA group, which decreased hydrogen bonding and consequently decreased protein adsorption [51], making cell detachment easier. Although the detachment rate of the MMC samples was slower than the non-MMC counterparts, a fully characterised poly(NIPAAM-co-NTBA) copolymer [52-54] allowed harvesting of dense and cohesive tissue-like modules as a continuous sheet within 45 minutes by a simple switch of temperature from 37 °C to 10 °C. Subsequent morphological analysis revealed that these dense and cohesive supramolecularly assembled living substitutes produced within 6 days in culture in the presence of FC had intact cell-cell and cell-ECM junctions and achieved similar level of biomimicry as scaffold and scaffold-free substitutes that were produced after 3 [55], 4 [25], 9 [56] or even 11 weeks [57] in culture. Further, although traditional self-assembled living substitutes require prolonged culture time to form and mature, in the presence of macromolecular crowding, such processes are enhanced, as evidenced by the presence of β-bands on the gels.

The transparency is an important factor in the development of an artificial corneal tissue. The light transmittance assay of developed ECM-rich HCFs sheets showed no significant difference in light transmittance in visible light range (380-780 nm)
between the produced crowded cell sheets and the PBS control and the non-crowded counterparts. It is speculated that the development of multilayered corneal stromal tissue using cell sheet technology in the presence of MMC (FC) will not further affect the light transmittance as the deposition of corneal ECM is organised in a similar fashion to the native tissue [25].

Further gene and protein analysis assays demonstrated that FC did not affect expression levels of collagenous proteins, glycoproteins, phenotypic markers and unintended differentiation marker. It is worth pointing out that both MMC and non-MMC counterparts were negative for CD34 and keratocan at protein level and very low expression at gene level. It has been reported that the expression of these markers rapidly declines in low-density cultures [58] after exposure to serum [37-39, 59] and as a function of time in culture [56, 60, 61].

The proteomics results of FC treated HCFs shows only a few proteins in the non-crowded control samples and abundance in protein deposition in the crowded samples (Table 2.2). Although this is in accordance with our theory, attention should be paid to this experiment, as it was run only once.
2.5. Conclusion

Here, we demonstrate that modulation of the *in vitro* microenvironment of HCFs with FC results in ECM-rich supramolecular assemblies within days rather than weeks or months in culture, without compromising fundamental cellular functions. This technology not only requires a lower cell number than multilayer cell sheets or high-density cultures, which is often not available, but also bypasses altogether such approaches that due to poor nutrient, oxygen and waste transport result in cell necrosis in the central cell-layers [62]. It further evades complex and expensive culture media that only temporarily will maintain phenotype, without actually increasing ECM deposition [63-67].
2.6. References


33. Lareu RR, Subrainhanya KH, Peng YX, Benny P, Chen C, Wang ZB, Rajagopalan R and Raghunath M. Collagen matrix deposition is dramatically


47. Mannello F and Tonti G. Concise review: No breakthroughs for human mesenchymal and embryonic stem cell culture: Conditioned medium, feeder layer, or feeder-free; medium with fetal calf serum, human serum, or enriched plasma; serum-free, serum replacement nonconditioned medium, or ad hoc formula? All that glitters is not gold! Stem Cells. (2007) 25: 1603-1609
Chapter 2


Chapter 3

Assessment of negative charged crowders on corneal fibroblasts culture

The contents of this chapter have been accepted for publication in Tissue Engineering: Part C as “Accelerated development of supramolecular corneal stromal-like assemblies from corneal fibroblasts in the presence of macromolecular crowders”. By Pramod Kumar, Abhigyan Satyam, Xingliang Fan, Yury Rochev, Brian J. Rodriguez, Alexander Gorelov, Lokesh Joshi, Michael Raghunath, Abhay Pandit and Dimitrios I. Zeugolis. 2015 Mar 12. [Epub ahead of print]
3.1. Introduction

The primary challenge of tissue engineering is to develop technologies that will allow engineering of tissue constructs reproducing the \textit{in vivo} cellular microenvironment. Traditionally, tissue engineering is based on the use of natural or synthetic polymer, in various physical forms, that provide a template for structural support and for cell attachment, growth, migration and finally neotissue development. However, scaffold-based approaches are often associated with immunogenicity and inflammation responses. Advancements in cell biology, polymer chemistry and engineering, along with better understanding of developmental and morphogenesis processes, have led to the development of bottom-up approaches that utilise the inherent proficiency of cells to create highly sophisticated tissue-like structures, termed tissue engineering by self-assembly / scaffold-free tissue engineering / cell sheet tissue engineering / modular tissue engineering [1-6].

Tissue engineering by self-assembly technologies have been extensively studied over the years for the development of implantable devices for various clinical targets, including skin [7], heart [8], blood vessel [9], lung [10], bone [11], liver [12], tendon [13] and cornea [14]. However, the major obstacle for wide acceptance, clinical translation and commercialisation of such technologies remains the prolonged culture time required to develop an implantable device, even in the presence of a scaffold (e.g. 84 days for corneal stromal layer) [15, 16]. Such prolonged \textit{ex vivo} culture is often associated with phenotypic drift and cell senescence [17, 18], which has triggered investigations into engineering more functional \textit{in vitro} microenvironments to maintain \textit{in vitro} cell phenotype and function [19-23]. Specifically to corneal repair, a number of studies have utilised complex culture systems to maintain corneal fibroblast phenotype, with variable degree of efficacy, but none of them has actually enabled accelerated extracellular matrix (ECM) production [24-31].

Herein, we hypothesise that macromolecular crowding (MMC), a biophysical phenomenon that regulates the intra- and extra- cellular milieu of multicellular organisms and increases thermodynamic activities and biological processes by
several orders of magnitude [32-37], will facilitate accelerated tissue-specific ECM deposition of human corneal fibroblasts (HCFs), whilst maintaining their function \textit{in vitro}. Although previous studies have demonstrated that the addition of inert macromolecules in the culture media, following the principles of excluding volume effect, increases the relative concentration of procollagen and proteinases, resulting in propeptide cleavage and subsequent accelerated ECM deposition in culture of skin and lung fibroblasts [38-40], there is no work to date on cells that are particularly susceptible to phenotypic drift, such as corneal fibroblasts.
3.2. Materials and methods

3.2.1. General materials
All tissue culture plastics were purchased from Sarstedt (Ireland) and Nunc (Denmark). All chemicals, cell culture media, reagents, carrageenan (CR) and dextran sulphate 500 kDa (DxS) were purchased from Sigma Aldrich (Ireland), unless otherwise stated. Live/Dead® cell viability kit and alamarBlue® cell metabolic activity kit were purchased from BioSource International (Ireland).

3.2.2. Human corneal fibroblast culture
HCFs (P10872, Innoprot, Spain) were cultured according to the supplier protocol. In brief, HCFs were seeded (5,000 cells/cm²) on poly(L-lysine) coated tissue culture flasks with fibroblast culture medium, buffered with HEPES and bicarbonate and 1.0 % penicillin / streptomycin. The cells were incubated at 37 °C with 5 % CO₂ / 95 % air in a humidified incubator up to confluency. The medium was replaced with fresh medium after 24 hours and changed after every 2-3 days. The cells used in all experiments were of 3-6 passages.

3.2.3. Macromolecular crowding
HCFs were seeded at 25,000 cells/cm². The medium was replaced after 24 hours with fresh medium containing the optimum concentration of macromolecular crowders (100 µg/ml of DxS or 75 µg/ml of CR; both negatively charged) [40, 41] with various concentrations of newborn calf serum (NBCS) or human serum (HS), ranging from 0.0 to 10 %. 100 µM L-ascorbic acid phosphate supplement was added in the medium to enhance collagen synthesis. The influence of MMC was assessed at 2, 4 and 6 days in culture.

3.2.4. Analysis of collagen deposition / densitometry
The medium and cell layer were digested separately with 150 µl pepsin (porcine gastric mucosa) solution containing phenol red (100 µg/ml pepsin in 0.05 N acetic acid) per well for 2 hours at 37 °C at continuous shaking (200 RPM) and neutralised with 1N NaOH. Samples for SDS-PAGE were prepared using appropriate dilution of
water and 5X sample buffer. Finally, 15 µl per sample solution per well was loaded on the gel (5 % running gel / 3 % stacking gel) after 5 minutes heating at 95 °C. Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad, Ireland) was used for the electrophoresis run. 50 V potential difference was applied for the initial 30 minutes, followed by 120 V for the remaining time. The gels were washed gently in ultra pure water and stained using SilverQuest™ (Invitrogen, Ireland) silver stain kit, according to the manufacturer instructions. Images of the gels were taken after brief washing with water. In order to quantify collagen type I, the gel densities (GeneTools software, Syngene, Ireland) of collagen α1(I) and α2(I) chains were evaluated and compared with the band densities of standard collagen type I (Symatese Biomateriaux, France).

3.2.5. Matrix metalloproteinase analysis
Gelatin zymography was used to assess matrix metalloproteinase (MMPs) activity. The culture media were collected after each incubation time and mixed with non-reducing SDS sample buffer (125 mM Tris-HCl, pH 6.8, 20 % glycerol, 2 % SDS, 0.002 % bromophenol blue). This was fractionated by SDS-PAGE using 10% gels containing 0.1% gelatin. Gels were washed with two incubations in 2.5 % Triton X-100 for 30 minutes. The gels were then incubated for 18 hours at 37 °C in a reaction buffer containing 50 mM Tris, pH 7.4, 5 mM CaCl₂, 1.0 µM ZnCl₂ to and finally stained with 0.5 % Coomassie G250 brilliant blue for 30 minutes. Gel images were taken after de-staining with 30 % ethanol / 10 % acetic acid. The developed gels bands were compared for relative expression of MMP2. Fresh medium with varying amounts of NBCS or HS were used as control.

3.2.6. Cell morphology
The morphology of HCFs was observed using phase contrast microscopy (Olympus IX81 inverted microscope, Japan) at X100 magnification.
3.2.7. Cell metabolic activity and cytotoxicity

The influence of MMC in cell metabolic activity and viability was evaluated using alamarBlue® and Live/Dead® assays. Briefly, 10 % alamarBlue® reagent was added into the various samples after aspiration of the culture medium and brief washing with PBS. The samples were incubated for 4 hours in a humidified chamber at 37 °C. After incubation, 100 μL of alamarBlue® reagent from the samples was transferred into a black 96-well plate. Fluorescence of this media was monitored using a microplate reader (Varioskan Flash, Thermo Scientific, Ireland) at excitation and emission 570 nm 600 nm respectively. The metabolic activity of the samples was calculated using the per cent reduction of dye, according to the supplier’s protocol and compared with the control samples having the same medium composition. Cell viability was determined using Live/Dead® viability kit (Invitrogen, Ireland). Briefly, the samples were incubated with calcein AM and ethidium homodimer solution (2 μM calcein-AM and 4 μM EthD-1) in PBS according to manufacturer’s staining protocol for 30 minutes. Afterwards, cell layers were washed with fresh PBS, for the removal of excess dye, and fluorescence images were taken using an Olympus IX81 inverted fluorescence microscope (Japan) using FITC and Texas red filter for live and dead cells respectively. Three images per sample were taken for live and dead cells.

3.2.8. Gene expression analysis

The total RNA was extracted using a modified Trizol isolation method. Briefly, cell layers were washed with PBS, after removing the medium, TriReagent® (Invitrogen, Ireland) was added and incubated for 5 minutes. The cell layer was mechanically disrupted using gentle pipetting. The phase separation was done using chloroform and the total RNA contained in the aqueous phase was purified using RNeasy® mini kit column (QIAGEN, Germany), as per supplier’s protocol. Three extractions were pooled at the end of the RNeasy protocol. Total RNA purity and quantity were evaluated using an ultraviolet spectrometer (NanoDrop ND-1000 Spectrophotometer, Thermo Scientific, Ireland). Reverse transcription of extracted mRNA was performed using MJ Research PTC-200 DNA Engine system (Promega...
RT System, UK), as per the manufacturers protocol. The prepared cDNA was evaluated using SYBR® Green master mix (QIAGEN, Germany) StepOnePlus™ Real-Time PCR System (Applied Bioscience, Switzerland). Each gene transcription was normalised to the transcription of housekeeping human 18S gene and $2^{-\Delta\Delta Ct}$ method was used to analyse the relative gene expression of target gene at various time points. The primers used for collagens, fibronectin, CD34, CHST6 and α-SMA are given in Table 3.1.

**Table 3.1.** | List of primers used for the qRT-PCR of various ECM molecules and HCFs markers

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<th>Target Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<td>Collagen Type Iα1</td>
<td>CTGTAACCTCCCTCCATCCC</td>
<td>GTCCATGAAAAATTTGCTCCC</td>
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<td>Collagen Type IIIα1</td>
<td>CTGGGGATGGAGCAAAAAC</td>
<td>AAAGCAACAGGGCCAAC</td>
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<tr>
<td>Collagen Type IVα1</td>
<td>ACGACATCATCAAAGGGGAG</td>
<td>ACCCACAATCTGTAACAC</td>
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<tr>
<td>Collagen Type Va1</td>
<td>ACCACCAAATTCTCGACC</td>
<td>CCTCAACACCTCTCTC</td>
</tr>
<tr>
<td>Collagen Type VIα1</td>
<td>ATCGGACCTAAGGGTGACC</td>
<td>TTCTCCCCTTTCACCCATC</td>
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<tr>
<td>Fibronectin 1(FN1)</td>
<td>GGACCAGGACCAACAAAAAC</td>
<td>AGACACTAACCACACT</td>
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<td>CCTCTTTTCTCCTTGCTTC</td>
</tr>
<tr>
<td>Cluster Differentiation molecules 34 (CD34)- Transcript Variant 1</td>
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<td>CACGTTTTACCAAGGACC</td>
</tr>
<tr>
<td>N-acetylglucosamine-6-O-Sulfotransferase-6 (CHST6)</td>
<td>TTAGGCGAGGAAAGGGAG</td>
<td>TCAAGGTTGAGAAAGCAGAG</td>
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3.2.9. Immunocytochemistry

For immunocytochemistry (ICC) staining, cells seeded in 4-well chambered Lab-Tek™ II slide and MMC treatment was carried out after 24 hours of cell seeding with fresh medium having crowder at concentration above. After incubation at various time points, the cell layer was fixed for 30 minutes with 3 % formaldehyde (freshly prepared from paraformaldehyde; PFA, Sigma) after PBS washing. The samples were washed three times in PBS. 3 % BSA for 30 minutes incubation at room temperature (RT) was used to avoid non-specific binding. Cell layers were then incubated with primary antibodies for 90 minutes at room temperature after dilution with PBS. The following primary antibodies were used: rabbit collagen type I, III, IV, V and VI (1:200; Abcam, UK); and mouse anti-fibronectin (1:200; Sigma Aldrich, Ireland). Mouse anti-actin, α-smooth muscle antibody (1:400 dilution), CD34 antibody (1:50 dilution) and keratocan antibody (1:50 dilution) were also used. For the detection of CD34, cold methanol fixing (cold methanol at -20 ºC for 5 minutes) was used after PFA fixation. The samples were incubated with secondary antibody for 30 minutes at room temperature after primary antibody incubation. The secondary antibodies used were Alexa Fluor® 488, chicken anti rabbit or donkey anti mouse against rabbit or mouse antibodies at the 1:400 dilutions (Invitrogen, Ireland). The antibody incubation was followed by three brief washes with PBS. For nuclear staining, DAPI (4′,6-diamidino-2-phenylindole) was used at 1:4000 dilution (Invitrogen, Ireland) after post fixation with 3 % PFA. Finally, the cover slips were mounted on glass slides with VectaShield (Vector Laboratories, UK) for direct observation. Images were taken using an Olympus IX81 inverted fluorescence microscope (Japan) with 10X objective. The fluorescence density of the images was assessed using Scope-Pro Plus software (Media Cybernetics, USA).

3.2.10. Cell sheet production

Thermal responsive polymer technologies were utilised to produce intact cell sheets. Briefly, 65 % N-isopropylacrylamide (NIPAAm) / 35 % N-tert-butylacrylamide (NTBA) copolymer [p(NIPAAm-co-NTBA)] was dissolved in absolute ethanol at 40 μg/ml and left for continuous shaking overnight. This polymer solution was
mixed with poly(L-lysine) (100 µg/ml) at 1:1 V/V ratio and kept under stirring overnight. 100 µl of the mixed solution was poured and spread evenly onto petri dishes, followed by incubation in an ethanol soaked desiccator overnight. The petri dishes were further dried in a 600 mBar vacuum oven at 40 °C for 3-4 hours. HCFs were seeded at 50,000 cells/cm² after UV sterilisation of the petri dishes for 2 hours. MMC treatment was carried out after 24 hours of cell seeding with fresh medium and 0.5 % HS. The culture dishes were kept in a humidified incubator at 37 °C and after 4 days of incubation, the intact cell sheets were detached from the petri dishes that were kept on a temperature-controlled plate at reduced temperature (10 °C) for the transition of polymer to hydrophilic state.

3.2.11. Cell sheet morphology (transparency) analysis
The morphology of HCFs cell sheets was evaluated using phase contrast microscopy at magnification X40 (Olympus IX81 inverted microscope, Japan).

3.2.12. Cell sheet light transmission analysis
After the crowding treatment, the cell layers were washed with PBS and 100 µl PBS was added in each sample well. A well with PBS only was used as control / blank. To maintain the zero absorbance and 100 % absorbance value, the unused wells and black dye were used; this black dye showed approximately 100 % absorbance, i.e. nearly nil transmittance, while the absorbance value in air (unused wells) was taken as 100 % transmittance as a reference. The optical density of the samples was measured using a spectrophotometer (Varioskan Flash, Thermo Scientific, Ireland) in visible light range (380-780 nm) wavelength with a resolution of 5 nm and the percentage transmission was calculated using this formula:

\[ \text{% Transmittance} = 100(10^A) \]

where A is absorbance measured.

3.2.13. Cell sheet atomic force microscopy analysis
HCFs were seeded in 4-well Lab-Tek™ II chamber slides at 25,000 cells/cm² and after 24 hours of seeding MMC treatment done as given above. After 4 days of
crowding treatment, cell layers were washed with PBS and fixed with 4 % paraformaldehyde at room temperature (RT) for 15 minutes. The samples were dehydrated using serial dehydration with 30 %, 50 %, 70 %, 90 % and 100 % ethanol after washing with 3 short PBS washes. Atomic force microscopy (AFM; MFP-3D, Asylum Research) was performed using rectangular Si cantilevers (SSS-NCH, Nanosensors, Switzerland) at nominal resonance frequency of 330 kHz and a spring constant of 42 N/m. AFM images were recorded using amplitude modulation mode in an ambient environment after drying out the samples using nitrogen gas.

At the end of culture time, samples were fixed in Bouin’s solution for 1 hour at 56 °C after fixation with PFA for 15 minutes. Samples were incubated in Weigert's iron haematoxylin staining for 10 minutes, after Bouin’s incubation and rinsing with running tap water. Then, the samples were placed under running tap water for 10 minutes and later were washed briefly with distilled water. Samples were stained in Biebrich scarlet-acid fuchsin solution for 12-15 minutes and washed again using the distilled water. The samples were differentiated using phosphomolybdic-phosphotungstic acid solution for 15 minutes. Subsequently, samples were transferred directly to aniline blue solution for 10 minutes. Samples were then exposed to 1 % acetic acid solution for 5 minutes, after brief rinsing with distilled water. The dehydration of the samples was performed quickly using 95 % ethanol, absolute ethanol (this step wipes off Biebrich scarlet-acid fuchsin staining) and cleared in xylene after short washing with distilled water. Finally, the images of the stained samples were taken using the IX81 microscope (Olympus, Japan) at 100X magnification, after mounting with resinous mounting medium.

3.2.15. Cell sheet Picro-sirius red staining
PFA fixed cell sheets were stained with Weigert’s haematoxylin for 8 minutes after several PBS washings and stained with 0.2 % phosphomolybdic acid hydrate, after rinsing in tap water. The cell-sheets were stained with picro-sirius red for 1 hour, followed by washing in acidified water. Dehydration was carried out using a series
of ethanol washes (70 %, 80 %, 90 %, 100 %). The final dehydration step was carried out with xylene for 5 minutes and the slides were mounted using DPX. Finally the images were captured with an Olympus IX-81 inverted microscope (Japan).

3.2.16. Statistical analysis
All results are presented as mean ± standard deviation. MINITAB™ (version 16, Minitab, Inc., USA) was used for statistical analysis. Two-sample t-test for pair wise comparisons and ANOVA for multiple comparisons were performed, after confirming that (a) the normal distribution of the samples (Anderson-Darling normality test); and (b) variances of the population were equal to one another (Bartlett’s and Levene’s tests for homogeneity of variance). If any of the above assumptions were violated, non-parametric statistics were used, with Mann-Whitney test for 2-samples and Kruskal-Wallis test for multiple comparisons. Each experiment was carried out in biological triplicates. Statistical significance was accepted for $p$ value < 0.05.
3.3. Results

3.3.1. Identification of optimal culture period and % new-born calf serum (NBCS) for maximum ECM deposition

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and supplementary densitometric analysis (Figure 3.1 & 3.2, respectively) revealed that in the absence of crowding molecules collagen remained in the media, whilst in the presence of crowding molecules collagen was deposited in the cell layer. At day 4, carrageenan (CR) induced the highest collagen deposition, independently of the NBCS concentration ($p < 0.001$; Figure 3.1 & 3.2). The enhanced matrix metalloproteinase (MMP) activity, as a function of increased NBCS concentration (Figure 3.3), resulted in no significant difference in collagen deposition between the different NBCS concentrations ($p > 0.05$), at a given time point (Figure 3.1 & 3.2). Collagen deposition was significantly increased from day 2 to day 4 and plateaued from day 4 to day 6 for a given NBCS concentration for non-crowded and crowded groups (Figure 3.1 & 3.2), as a function of MMP activity (Figure 3.3). Phase contrast microscopy revealed that HCFs maintained their spindle morphology for all NBCS concentrations, time points and crowding molecules (Figure 3.4). No significant difference ($p > 0.05$) in cell metabolic activity (Figure 3.5A) and cell viability (Figure 3.5B) was observed, as a function of crowding molecule, NBCS concentration and time in culture.
**Figure 3.1.** SDS-PAGE analysis revealed that in the absence of crowders, collagen type I remained in the media, whilst in the presence of crowders, collagen type I was deposited in the cell layer. 0.0 to 10% indicates NBCS concentration. Crowding effect was induced using 100 \( \mu g/ml \) DxS and 75 \( \mu g/ml \) of CR.
Figure 3.2. Densitometric analysis of SDS-PAGE (Figure 3.1) demonstrated the increased collagen type I deposition in the cell layer in the presence of crowders. The highest collagen type I deposition was detected in the presence of CR after 4 days in culture in the presence of 0.5% NBCS. Note: Collagen α1(I) and α2(I) chains were evaluated and compared with the band densities of standard (STD) collagen type I (100 µg/ml). NBCS was used for this set of experiments.
Figure 3.3. | Gelatin zymography indicated enhanced MMP activity as a function of increased serum concentration at a given time point. NBCS was used for this set of experiments.
Figure 3.4. | Phase contrast microscopy revealed that HCFs maintained their spindle shape morphology, independently of the serum concentration and crowder present.
Figure 3.5. Cell metabolic activity (A) and cell viability (B) was not affected as a function of time in culture, crowder present and serum concentration.
3.3.2. Influence of serum origin in ECM deposition

SDS-PAGE (Figure 3.6) and supplementary densitometric analysis (Figure 3.7A) revealed significantly higher collagen deposition in the presence of human serum (HS; \( p < 0.001 \)) at all time points, independently of the crowder used, which was attributed to the enhanced MMP activity in the presence of NBCS (Figure 3.7B). The highest collagen deposition was detected after 4 days in culture, in the presence of CR \( (p < 0.001; \) Figure 3.7A). Complementary immunocytochemistry (ICC) analysis further corroborated the enhanced collagen and fibronectin deposition in the presence of crowders (Figure 3.7C). Phase contrast microscopy revealed that HCFs maintained their spindle morphology at all time points, independently of the crowder used or serum origin (Figure 3.8). No significant difference \( (p > 0.05) \) in cell metabolic activity (Figure 3.9A) and cell viability (Figure 3.9B) was observed between NBCS and HS, independently of the crowder used and time in culture.
**Figure 3.6.** SDS-PAGE analysis of HCFs deposited collagen type I, indicated that in the presence of HS, similar to the presence of NBCS, enhanced collagen type I deposition was observed in the presence of crowders, whilst in their absence, collagen type I remained in the media.
Figure 3.7. MMC accelerates ECM deposition in HCFs culture in the presence of NBCS and HS. Densitometric analysis of SDS-PAGE confirmed the high collagen type I deposition in the cell layer as early as 2 days in culture, with the highest deposition after 4 days in culture in the presence of HS (A). MMP-2 expression was higher in the presence of NBCS, as opposed to HS and an increased MMP-2 activity was evidenced as a function of time in culture (B). ICC analysis further corroborated the enhanced collagen type I and fibronectin deposition in the presence of DxS and CR (C).
Figure 3.8. | Phase contrast microscopy revealed that HCFs maintained their spindle shape morphology for all time points (2, 4 and 6 days), irrespective of the crowder and sera used.
Figure 3.9. | Cell metabolic activity (A) and cell viability (B) was not affected, independently of the crowder and sera used and time in culture. More than 80% of HCFs cells were viable in the presence of Dxs as well as CR.
3.3.3. Evaluation of MMC on gene expression of HCFs

Gene expression analysis (Figure 3.10) indicated that dextran sulphate (DxS) significantly upregulated the expression of collagen type I, collagen type V, CD34 and $\alpha$-SMA and significantly downregulated collagen type VI at all time points ($p < 0.001$). Using CR, no significant difference in gene expression profile was detected at day 6 (longest time point; $p > 0.05$; Figure 3.10).
Figure 3.10. | Macromolecular crowders preferentially affect gene expression profile of ECM proteins and cell markers. qRT-PCR analysis demonstrated that CR did not affect the gene expression profile of molecules assessed at day 6, whilst DxS significantly increased gene expression of collagen type I, collagen type V, CD34 and α-SMA at all time points tested.
3.3.4. Evaluation of MMC on protein deposition of HCFs

ICC analysis (Figure 3.11) and complementary densitometric analysis (Figure 3.12) demonstrated enhanced deposition of collagenous proteins (types I, III, IV, V and VI) and glycoproteins (fibronectin) under MMC conditions, with no difference in α-SMA expression. Independently of the crowder used and the time in culture, HCFs were negative for keratocan and CD34 (Figure 3.13).
Figure 3.11. MMC enhanced ECM deposition in the cell layer of HCFs. ICC further confirmed the enhanced deposition of tissue-specific ECM in the presence of macromolecular crowders, whilst myofibroblast trans-differentiation marker (α-SMA) was not expressed.
Figure 3.12. Relative immunofluorescence intensity of ICC analysis confirmed the high deposition of collagenous proteins (type I, III, IV, V, VI) and glycoproteins (fibronectin) under MMC conditions, whilst no difference was observed for myofibroblast trans-differentiation marker (α-SMA).
Figure 3.13. ICC analysis indicated that cells were negative for CD34 and keratocan, independently of the time in culture and presence or absence of crowding molecules.
3.3.5. ECM-rich HCFs sheets production and characterisation

The abundant ECM deposition prohibited detachment of intact HCF sheets (Figure 3.14A) from commercially available N-isopropylacrylamide (pNIPAAM) coated dishes, although cell attachment and growth was not affected. Coated dishes with an N-isopropylacrylamide copolymer [65 % pNIPAAM / 35 % N-tert-butylacrylamide (NTBA); p(NIPAAM-co-NTBA)] allowed attachment and growth of HCFs in the absence (Figure 3.14B) and presence (Figure 3.14C) of CR. Time-lapse microscopy showed a slower detachment rate of the MMC HCFs sheets rather than their non-crowded counterparts, due to the abundant ECM deposition induced under MMC conditions (Figure 3.14D and Figure 3.15). Complete detachment of HCFs sheets was accomplished within 45 minutes at 10 °C; MMC produced HCFs sheets did not shrink and maintained their structure, due the abundant deposited ECM (Figure 3.14E). Histological analysis using Masson’s trichrome staining (Figure 3.16A) and Picro-Sirius red (Figure 3.16B) and ICC analysis for collagen type I (Figure 3.16C) and collagen type VI (Figure 3.16D) further corroborated the enhanced ECM deposition HCFs living substitutes under MMC conditions. No significant difference ($p > 0.05$) in transparency between PBS buffer, control surface, non-MMC and MMC HCFs living substitutes was detected (Figure 3.16E). Atomic Force Microscopy (AFM) analysis revealed the presence of ECM in the intercellular regions of the HCFs living substitutes produced under MMC conditions (Figure 3.16F).
Figure 3.14. | HCFs sheet production. The ample ECM deposition prohibited detachment of intact cell sheets from commercially available pNIPAAM coated dishes (A). Cell culture dishes coated with a p(NIPAAM-co-NTBA) temperature-responsive copolymer allowed attachment and growth of HCFs in the absence (B) and presence (C) of CR. Time-lapse microscopy demonstrated that due to enhanced ECM deposition, MMC-produced HCFs sheets had a slower detachment rate than their non-crowded counterparts (D). The abundant deposited ECM in the presence of crowders prohibited cell sheet shrinkage following detachment (E).
Figure 3.15. Time-lapse microscopy revealed that in the presence of CR, a slower detachment rate was observed compared to the non-crowded counterparts.
Figure 3.16. | HCFs sheet characterisation. Histological analysis with Masson’s trichrome (A) and Picro-Sirius red (B) staining further demonstrated the enhanced ECM deposition in the presence of CR. ICC for collagen type I (C) and collagen type VI (D) further confirmed the corneal-specific ECM deposition. No significant difference in % transparency between MMC HCFs sheets and PBS, control surface and non-MMC sheets was observed at 0.5 % HS after 4 days in culture (E). AFM analysis also confirmed ECM deposition in the presence of CR at 0.5% HS after 4 days in culture (30 nm height scale) (F)
Chapter 3

3.4. Discussion

Cell-based therapies encompass removal of cells from their optimal in vivo setting and expansion in vitro to attain sufficient numbers and to subsequently develop a tissue substitute. However, cell growth, in the still primitive in vitro microenvironment, is often associated with phenotypic drift and cell senescence. It is therefore imperative to develop more functional in vitro microenvironments to enable clinical translation and commercialisation of cell-based therapies. Herein, we assessed the bi-facet potential of MMC in tissue engineering and regenerative medicine: firstly to enable the accelerated production of tissue-specific ECM-rich living substitutes by promoting the supramolecular assembly of cellular secretome in higher order tissue-like modules; and secondly to allow in vitro propagation of cells, whilst maintaining their native functions.

SDS-PAGE, densitometry and ICC analysis demonstrated that MMC significantly enhanced ECM deposition, with over 10- to 12-fold increase after 4 days in culture in the presence of CR and 0.5 % NBCS and 0.5 % HS respectively. This is in accordance to previous studies that demonstrated that negatively charged macromolecules (e.g. Dxs and CR) accelerate tissue-specific ECM deposition, recognising CR as the most effective crowder, due to its inherent polydispersity that achieves more effective volume occupancy / exclusion and thus higher ECM deposition [40].

The constant or not increased ECM deposition as a function of serum concentration, serum origin and time in culture was attributed to the enhanced proteolytic activity that resulted in degradation of deposited ECM, independently of the crowding molecule used. Although it would have been expected α2 macroglobulin, a known MMP inhibitor present in sera, to suppress MMP activity, previous studies have demonstrated an increased MMP activity in both animal and human serum as a function of increased serum concentration [40], which appears to overwhelm α2 macroglobulin activity. This finding is of significant importance as: (a) high serum concentration increases development costs; (b) prolonged exposure to serum results in phenotypic drift of corneal cells in culture [27, 42]; and (c) the use of human serum (HS), as opposed to animal sera, can avoid potential interspecies transmission
of disease and severe immune reactions, supporting further its use for the development of clinically relevant cell therapies [43-51].

Subsequent gene analysis for ECM proteins and cell markers revealed that CR did not affect the gene expression profile of HCFs at day 6, whilst significant upregulation in collagen type I, collagen type V, CD34 and α-SMA was observed in the presence of DxS. This observation, with respect to DxS, can be attributed in two, rather contradictory, theories. The first theory is that DxS promotes physiological tissue formation, given that collagen type V is a regulatory fibril-forming collagen that is co-localised with collagen type I in human cornea and plays a pivotal role in fibril and ECM assembly during tissue development and growth, whilst dysfunctional regulation results in loss of transparency [52-54]. This theory is also supported by the observed upregulation of CD34, downregulation of which is associated with a myofibroblast differentiation, cornea pathophysiology and scarring [26, 55, 56]. The second theory is that DxS promoted trans-differentiation to a myofibroblast phenotype, as evidenced by upregulation of α-SMA and collagen type I [57-59]. Moreover, DxS has been used previously as means to create an in vitro scar model [60], which further enhances this notion. In any case, SDS-PAGE demonstrated that in the presence of CR, collagen type V was deposited, which indicates that CR supports a more physiological tissue formation.

ICC analysis demonstrated that HCFs were negative for α-SMA, CD34 and keratocan. This is not surprising, given that the expression of these markers rapidly declines in low-density cultures [61], after exposure to serum [62-64] and as a function of time in culture [65-68]. Nonetheless, this is of significant importance as CR may be further investigated for regenerative purposes, whilst DxS may be used for drug discovery purposes, further enhancing the multifaceted potential of MMC.

The abundant deposition of ECM, in the presence of CR, prohibited detachment of intact HCFs sheets using commercially available pNIPAAM dishes. However, a fully characterised p(NIPAAM-co-NTBA) copolymer [69-72] afforded complete detachment of dense, cohesive and structurally aligned corneal stromal tissue modules within 4 days in culture with intact cell-cell and cell-ECM junctions and high % transmittance, similar to native corneal stromal and previously produced scaffold and scaffold-free equivalents [73-76]. This enhanced deposited ECM as a
result of MMC overcomes the need of high cell populations, often not available and multi-layer cell sheets that due to poor nutrient transport and waste accumulation result in cell necrosis in the central layers [77].

It is reported that dextran could not cross the biological membrane which make it ideal candidate as a markers and probes of specific subcellular compartments [78]. The cell membrane permeability of CR has not been reported. The CR is also negatively charged macromolecules similar to the DxS, which will prohibit the entry of CR within the cell membrane. In current study the medium containing either DxS or CR was discarded and cell layer / cell sheets were washed using Hank’s balance salt solution (HBSS) before any further study. Thus, it is assumed that there is no incorporation of DxS or CR within the HCFs cells or developed cell sheets. Nonetheless, a more detailed study should be conducted to actually quantify the amount of these molecules after crowding.
3.5. Conclusion

In summary, MMC resulted in fast (4 days) and enhanced (10- to 12- fold) tissue-specific ECM deposition in HCFs culture, as opposed to traditional methods that require months in culture. Morphological and protein analysis confirmed corneal stromal-like architecture and composition. Gene analysis indicated that different crowding molecules preferentially drive HCFs phenotype in culture with tremendous potential in regenerative medicine and drug discovery. Overall, MMC provides an alternative approach in the field of cell-based therapies with considerable clinical implications.
3.6. References


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45. Mannello F and Tonti G. Concise review: No breakthroughs for human mesenchymal and embryonic stem cell culture: Conditioned medium, feeder layer, or feeder-free; medium with fetal calf serum, human serum, or enriched plasma; serum-free, serum replacement nonconditioned medium, or ad hoc formula? All that glitters is not gold! Stem Cells. (2007) 25: 1603-1609


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74. Guo X, Hutcheon AEK, Melotti SA, Zieske JD, Trinkaus-Randall V and Ruberti JW. Morphologic characterization of organized extracellular matrix


Chapter 4

Summary, future directions and conclusions
Chapter 4

4.1. Introduction
The limited availability of donor corneal tissue that only partially will restore corneal function is the major limitation of corneal grafting. Bioengineering approaches have also failed to closely replicate the highly ordered environment of corneal tissue. Tissue engineering by self-assembly emerged as a potential approach to create supramolecular assemblies that closely imitate native tissues; however the prolonged time required to develop an implantable device results in phenotypic and therapeutic potential loss [1-3]. The overall objective of the present study was to assess the effect of macromolecular crowding (MMC) on corneal fibroblasts for the accelerated native extracellular matrix (ECM) deposition, whilst maintaining cell phenotype.

4.2. Summary
Herein, we demonstrated that the use of macromolecular crowding in human corneal fibroblasts with neutral FC (Ficoll® 400kDa and Ficoll® 70kDa) or negatively charged polydispersed macromolecules (dextran sulphate 500kDa or carrageenan) accelerate the deposition of corneal stromal specific ECM within 2-6 days in vitro culture. It was observed that the ECM deposition was significantly higher in the presence of low serum supplementation, due to the low matrix metalloproteinase content. Human serum induced higher ECM deposition than animal sera, again due to the lower content of matrix metalloproteinases. Genomic study confirmed that there is no change in gene expression for various ECM molecules, corneal stromal markers (CHST 6 & CD34) or de-differentiation marker (α-SMA) in the presence of Ficoll® and carrageenan. Dextran sulphate, on the other hand, resulted in significant increase in collagen type I, collagen type V, CD34 and α-SMA genes, suggesting that carrageenan and Ficoll® can be used for reparative purposes, whilst dextran sulphate can be utilised for the development of pathophysiology models. Proteomic analysis and immunocytochemistry further confirmed this observation. The abundant ECM deposition in the presence of MMC prohibited cell sheet detachment using commercially available poly(NIPAAM) coated dishes. However, poly(NIPAAM-co-NTBA) copolymers supported cell growth and enabled complete detachment of dense and cohesive corneal stromal-like tissue within 4-6 days in culture. The
developed tissue substitute showed high degree of light transmittance and structural organisation, similar to native corneal stromal tissue [4-7].

Herein, we provide evidence that the modulation of the in vitro microenvironment of human corneal fibroblasts with MMC facilitates the production of a dense and cohesive corneal stromal-like tissue substitute within days rather than weeks or months in culture, without compromising fundamental cellular functions. This work provides a new perspective in the field of tissue engineering by self-assembly with significant clinical implication.

4.3. Limitations

The major limitation of this work is that we assessed the influence of macromolecular crowding on human corneal fibroblasts and not on human corneal keratocytes, as we could not get access to human cells. We decided not to use animal cells as, there are not sufficient markers available for bovine cells and we would have needed several rat / mice corneas. Further, we would have had to repeat all this study with human cells to demonstrate clinical relevance.

A second limitation that we ought to identify is that the work conducted using conventional ex vivo culture conditions (e.g. far from physiological surface topography and substrate stiffness), which may have altered cell phenotype. Further, although an extensive in vitro study was conducted, the produced tissue-equivalents should be characterised in vivo to ensure safety and clinical relevance.

4.4. Future directions

The development of a bioengineered corneal tissue is still at investigatory stage [8]. Modulation of in vitro microenvironment of corneal keratocytes and corneal fibroblasts is a potential strategy to design a well-defined corneal-stromal substitute with native tissue-like architecture. Although this work demonstrated the potential of macromolecular crowding in corneal stromal regeneration, other complementary in vitro microenvironment modulators should also be considered (e.g. surface topography, substrate stiffness, oxygen tension) for functional repair and regeneration. The following sections outline potential routes towards this goal.
4.4.1. Macromolecular crowding of primary corneal keratocytes

The corneal fibroblasts used in current study, are activated keratocytes and thus are not the authentic representative of the *in vivo* stromal tissue. Thus, several questions remain open, and should be studied further. The corneal fibroblasts used in present study, did not express the keratan sulphate containing proteoglycans, which play an important role in fibrillar assembly in corneal stromal tissue [9]. Further, the exact mechanism of ascorbate-induced keratan sulphate proteoglycans synthesis and deposition requires further analysis. It is possible that the higher deposition of collagen in keratocytes layer may binds to integrin receptors on the cellular surface, which can favour the upregulation of keratan sulphate proteoglycans. Thus, future studies may seek to focus on these observations, and determining the sequence of events taking place, which may have potential significance in the development of nascent corneal stromal tissue.

4.4.2. Macromolecular crowding in the presence of hypoxia

The use of biochemical factors, such as physiological hypoxic conditions, during *in vitro* culture is a well-defined approach to emulate the *in vivo* microenvironment [10, 11]. In corneal stromal culture, low oxygen tension has been shown to reduce myofibroblast differentiation of corneal keratocytes and to increase ECM synthesis [12-14]. Thus, the use hypoxic conditions in addition to MMC will further enhance ECM deposition, whilst maintaining keratocyte phenotype in culture.

4.4.3. Macromolecular crowding on substrates with physiologically relevant topography and rigidity

Previous studies have demonstrated that modulation of the *in vitro* microenvironment with topographical features results in cell and extracellular matrix alignment [15] and phenotype maintenance of corneal keratocytes [16, 17]. Indeed, the selection of topographical cues is an important parameter for the keratocytes phenotype maintenance in culture [15, 18, 19]. The contact guidance of corneal stromal cells imitate the native cytoskeleton orientation, overall cell shape maintenance, cell differentiation and ECM alignment [17, 20, 21]. This arrangement also supports the alignment of secreted ECM deposition and affects the transparency
4.5. Conclusions

The current study uses macromolecular crowding as a means to modulate the in vitro culture of human corneal fibroblasts for the development of extracellular matrix rich corneal stromal equivalents. We identified that Ficoll® and carrageenan are suitable crowding molecules for enhanced extracellular matrix deposition and cell phenotype maintenance. Dextran sulphate, on the other hand, although also promoted enhanced extracellular matrix deposition, induced trans-differentiation towards myofibroblast lineage, suggesting that Ficoll® and carrageenan can be used for reparative purposes, whilst dextran sulphate can be used for the development of pathophysiology models. We also identified potential multifactorial approaches (e.g. combination of macromolecular crowding with physiologically relevant surface topography, substrate stiffness, oxygen tension) that may lead to the development of functional scaffold-free corneal substitutes.
4.6. References

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Chapter 5

Appendices
5.1. List of reagents / chemicals used.

Table 5.1. | List of reagents / chemicals used

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## Reagents/ Chemicals

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### 5.2. List of instruments used.

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<td>MJ Research, UK</td>
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<td>Mini-protean tetra electrophoresis system</td>
<td>Bio-Rad, Ireland</td>
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<td>DMLS2 phase contrast microscope</td>
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<td>Heraeus Fresco 17(^\text{®}) Centrifuge</td>
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<td>BSC Class II BSC Fischer</td>
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<td>Vortex (FB15012)</td>
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<td>Media Cybernetics Products, USA</td>
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<td>Heating mantle</td>
<td>Techni DRI Block DB-2A, UK</td>
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<td>Water filter assembly (BERNSTEAD EASYPURE II)</td>
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<td>Electrophoresis power supply (EPS200)</td>
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<td>Weighing scale MH 124</td>
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<td>Gel scanner (Scanjet 7400)</td>
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<td>q-PCR Stepone Plus</td>
<td>Applied Bioscience, Switzerland</td>
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<tr>
<td>Gal casting tray and combs</td>
<td>Bio-Rad, Ireland</td>
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<tr>
<td>Tran illuminator</td>
<td>(G-Box, Syngene, UK)</td>
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</table>
5.3. Protocols used in the study

5.3.1. Cell culture

5.3.1.1. Cell thawing and culturing

- Wear protective gloves and face shield and take out the tube containing the frozen cells from liquid nitrogen cylinder.
- Thaw the contents of tube by rubbing in palm or water bath at 37 °C.
- Transfer the contents of the tube in a 15ml falcon tube containing 10ml of appropriate media inside the laminar air flow hood.
- Centrifuge the tube at 1500 RPM for 5 minutes.
- Remove the supernatant and discard it.
- Add 1ml of pre-warmed culture media in to the same falcon tube and mix properly with gentle aspiration to distribute the cells homogeneously.
- Count the cells using haemocytometer.
- Transfer 1ml of the cell suspension (known cell density) to a new T75 flask or as appropriate.
- Add 9ml or appropriate amount of culture media (fibroblast medium; Innoprot).
- Label the flasks with name date and cell type and passage no. etc.
- Incubate the cell’s flask in humidified incubator at 37 °C and 5% CO2/95% air.
- Change the fresh media every 2-3 days or as per requirement.
- Monitor cells using phase contrast microscope (Leica).
- If cells are starting to float or media colour changed, take off old media and rinse the cells using warm DMEM.
- Add fresh appropriate culture media.
- Monitor cells for confluence.
- When cells are ready to split (80-90%) take off the media.
- Rinse the cells using 10ml of DMEM and pour off the DMEM.
- Add 5ml of trypsin/EDTA and either incubate at 37 °C or room temperature for 5minutes.
- When trypsin start to lift off the cells from the flask, tap the bottom of flask gently and bring cells in a corner of flask.
- Use cell scraper, if needed.
5.3.1.2. Cell counting

- Trypsinise the cells as above.
- Add equal amount of serum containing media, aspirate several time and transfer the cell suspension to a new falcon tube.
- Ensure the haemocytometer is clean using 70% ethanol.
- Take 10µl of cell suspension and add 10µl of trypan blue and mix them properly.
- Add 10µl cell suspension on each side of haemocytometer under the cover slip.
- Allow the sample to be drawn out of the pipette by capillary action, the fluid should run to the edges to the grooves only.
- Focus on the grid lines of haemocytometer using 10X objective of the microscope.
- Focus on one set of 16 corners square as indicated by the circle in Figure 5.1.
- Count the cells in this area of 16 squares.
- Count only healthy cells unstained by trypan blue.
- Count the cells that are within the square and any positioned on the right hand or bottom boundary line.
- Dead cells stained blue with trypan blue can be counted separately for viability count.
- Move the haemocytometers to another set of 16 corner square and continue the counting until all 4 sets of 16 corners are counted.
Get the average count and then multiply by two to adjust for the 1:2 dilution in trypan blue.

This is equivalent to number of cells $\times 10^4$ per ml.

Finally total number of cells can be obtained by multiplying the above number by the total volume of cells suspension used.

**Figure 5.1.** Haemocytometer configuration.

5.3.1.3. Cell freezing

- Trypsinise the cells as above and centrifuge to form a cell pellet.
- Re-suspend the pellet in adequate amount of freezing medium (10% DMSO + 90% of HCFs media; DMEM/F12 + 5ng/ml of FGF-2 + 10% NBCS+ 1% P/S).
- Pipette up and down several times gently to ensure homogenous suspension.
- Dilute this cell suspension so as to make a cell suspension of $1 \times 10^5$ cells per ml.
- Transfer 1ml of cell suspension to each freezing vial to adjust $1 \times 10^5$ cells per vial.
- Label each tube with date cell name, passage and initials of the name.
- Transfer the vial to -80 °C overnight in MR frosty for 24 hours and then to liquid nitrogen.
5.3.1.4. Cell seeding for *in vitro* study

- Preheat culture media for 30 minutes in a 37°C water bath.
- One confluent T75 flask is needed for three 24 well plate or more (depends on cell density).
- Trypsinise and count the cells as above.
- Add the cell suspension in appropriate medium to dilute the cell density as required (50,000 cells/ml).
- Preheat the cell suspension again in water bath at 37°C.
- For Live/Dead® assay keep the poly(L-lysine) coated glass cover slip inside the 24 well plates before cell seeding.
- In a similar way, the cells are seeded on Lab-Tek™ II plate for immunocytochemistry
- Seed the cells according to 25,000 cells/cm² area or as needed.
- Incubate cell culture in humidified chamber at 37 °C at 5% CO₂ level for 24 hours.
- The culture is ready for further experiment after this time period.

5.3.2. Macromolecular crowding

- Seed the HCFs as above at 25,000 cells/cm² using human corneal keratocytes medium composed of DMEM/F12 + 10% of new born calf serum (NBCS) +/− 5ng/ml of FGF 2 and 1% penicillin/streptomycin.
- Allow the cells to settle in culture at 5% CO₂/ 95% air at humidified chamber at 37 °C for 24 hours.
- After 24 hours of cell seeding replace the medium with fresh medium containing 100µM of L ascorbic acid and optimum amount of macromolecules; DxS 100µg/ml: CR, 75µg/ml and Ficoll®70 + Ficoll®400: 37.5mg/ml + 25mg/ml.
- The serum concentration used with macromolecular crowding may vary from 0.0, 0.5, 1.0, 2.0, 5.0 and 10% NBCS or 0.5% of human serum.
- After macromolecular crowding treatment the culture was allowed for various time points i.e. 2, 4 and 6 days without changing the medium in a humidified incubator at 37 °C at 95% air / 5.0% CO₂.
5.3.3. Evaluation for collagen type I

5.3.3.1. Preparation of pepsin solutions and sample digestion

- Make the pepsin solution just before the adding in the samples (1mg/ml in 0.5M acetic acid) -pepsin solution A.
- Dilute pepsin solution A to 100µg/ml using the HBSS- pepsin solution B.
- Collect the medium sample in pre-labelled centrifuge tube and keep at 4 °C.
- Wash the cellular layer briefly using the DPBS.
- Add pepsin solution A (1 mg/ml) in medium sample (100 µl to 900 µl).
- Add pepsin solution B in cell layer (100µl/well in 24 well plate).
- Keep medium and cell samples in rotating shaker at 37°C for 2 hours with continuous shaking at 200 RPM.
- Scrape off cell layer using tips and collect in pre labelled test tubes after shaking.
- Pool the samples of three cell layer wells in one test tube.
- These samples (medium as well as cells) can be kept at cold condition (2-8 °C) for longer time for SDS-PAGE run.

5.3.3.2. Sample preparation for SDS-PAGE

**Collagen standard:** Dissolve 1mg type I collagen (Symatese) in 1ml 0.5M acetic acid or dilute it with HBSS according to the need, up to 100µg/ml.
- Add 5 µl of phenol red reagent in cell layer tubes and collagen standard.
- Neutralise the cell samples, medium samples and standard collagen sample by step-wise addition of 1N NaOH Solution. The solution colour will turn pink at neutralisation.
- Take the cell samples, medium samples and collagen standard in a fresh centrifuge tube (24µl each or appropriate).
- Add 24µl of double distilled water in these samples (or appropriate).
- Add 12µl of 5X sample buffers to get 1:5 dilution (sample to 5X buffer).
- Vortex the samples well and centrifuge them briefly. Store them at 4 °C.
- Prior to use, heat the samples at 95°C for 5minutes to denature the proteins and standard, vortex and centrifuge briefly the samples again.
- Load 15 µl sample per well in a well of stacking gel for SDS-PAGE run.
5.3.3.3. Sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE)

5.3.3.3.1. Materials/reagents required

- **1.875M Tris-HCl, pH 8.8:** Dissolve 22.70g Tris-base (Bio-Rad, 161-0716) in 80ml ddH₂O; add 2ml concentrated HCl (37%), leave it overnight to equilibrate, adjust pH to 8.8 with a few drops concentrated HCl, make it up to 100ml with ddH₂O. Keep it at 4-8 ºC.
- **1.25M Tris-HCl, pH 6.8:** Dissolve 15.14g Tris-base in 70ml ddH₂O; add 7ml concentrated HCl (37%), leave it overnight to equilibrate, adjust pH to 6.8 with a few drops concentrated HCl, make it up to 100ml with ddH₂O. Keep it at 4-8 ºC.
- **5x sample buffer:** Dissolve completely 0.25g SDS (Bio-Rad 161-0301) in 0.625ml 1.25M Tris-HCl, pH 6.8 and 2ml ultra pure water. Leave it overnight for the foam to settle. Top up with glycerol (Bio-Rad) to 5ml (approximately 2.3ml). Add 2.5mg bromophenol blue (Bio-Rad 161-0404) per 10ml buffer.
- **5x running buffer:** Dissolve 15.1g Tris-base (Bio-Rad 161-0716), 72g glycine (Bio-Rad 161-0718) and 5g SDS (Bio-Rad 161-0301) in 1lt ddH₂O. Store at 4ºC. 1x running buffer is made to run the gel from 5x running buffer by diluting in ddH₂O. Alternatively, 10X Tris-Glycine-SDS 5lt tube can be purchased (Bio-Rad 161-0772). 1x running buffer is made to run the gel from 10X running buffer by diluting in ddH₂O.
- **30% Acrylamide/Bis (37.5:1) (Bio-Rad, 161-0158).**
- **10% SDS (Bio-Rad, 161-0416).**
- **100mg/ml Ammonium Persulphate (Bio-Rad, 161-0700) in ddH₂O.** Dissolve 500mg APS in 5ml ddH₂O, aliquot it in eppendorf tubes and keep it at – 20ºC.
- **TEMED (Bio-Rad, 161-0800).**
- **10% and 70% Ethanol in dH₂O**
5.3.3.3.2. Procedure for making the gels

- Take out an aliquot of 10% SDS and APS from -20°C to thaw (put it back at -20 °C at the end).
- Clean glass plates with 70% ethanol and wipe dry with microscope tissue papers.
- Set the gel making apparatus ensuring that the glass plates fit snugly to the platform (mini gel: 1mm space using appropriate spacers).
- Check for any leaks by pouring water prior to making the gels.
- Add the gel ingredients to make the 5% resolving gel. This can be done in a 15ml or 50ml conical falcon tube (*add APS & TEMED at last*).
- Using a Pasteur pipette, pour the prepared mixture carefully into the space between the 2 glass plates to reach about 1 cm (mini gel) from the bottom of the wells etched out by the comb (keep the excess solution to check how quickly the gels will be polymerised).
- Overlay the gel with 10% ethanol solution to cut off oxygen in contact to the gels.
- Leave it aside for approximately 30 minutes (check with remaining solution).
- During this settling period, prepare the 3% stacking gel (*do NOT add APS & TEMED until the gel solution is ready for pouring*).
- A line at the ethanol-gel interface that initially had disappeared will reappear after the 30 minutes period indicating that polymerisation is complete.
- Carefully aspirate the ethanol out of the glass plates using a syringe and imbibe any traces using filter paper.
- **Now add the APS and TEMED** to the stacking gel solution and carefully pour it on top of the polymerised resolving gel. Immediately insert the comb taking care to avoid trapping any air bubbles (keep the excess solution to check how quickly the gels will be polymerised).
- Allow it to set for 10 - 15 minutes for gelling.
5.3.3.3. SDS-PAGE run

- After the gels have been set, remove the combs from the gels slowly.
- Assemble the electrophoresis apparatus, for small gel apparatus fit the gel plates on the electrode bar and fit the set into the inner chamber and clamp them.
- Fill the upper/inner chamber with 1X running buffer.
- Wash the wells by squirting buffer into the wells with a hypodermal needle syringe to remove all air bubbles.
- Load the standards, samples and markers using Hamilton syringe (15 µl).
- Wash the syringe in between using the running buffer in the chamber (at least 5-times).
- Put the upper chamber on the main chamber, close the lid and start the current to run the gel(s).
- For the mini gel→ run at constant voltage: 50V until the front reaches the end of stacking gel (± 30-40 minutes), then 120V until the front reaches the end of the separating gel (> 1 hour).
- Remove the glass using the wonder wedge, cut the lower right hand corner and release the gel slowly into ddH₂O.
- Proceed silver staining (as per the recommendation of SilverQuest®, Invitrogen Protocols).
5.3.3.4. Silver staining

Follow the steps below for silver staining of SDS-PAGE gels.

<table>
<thead>
<tr>
<th>Step</th>
<th>Reagent</th>
<th>Volume</th>
<th>Incubation Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixing</td>
<td>Ethanol 40 ml, acetic acid 10 ml and ultra pure water to 100 ml</td>
<td>100 ml</td>
<td>20 minutes</td>
</tr>
<tr>
<td>Washing</td>
<td>Ethanol 30 ml, ultra pure water to 100 ml</td>
<td>100 ml</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Sensitizing</td>
<td>Ethanol 30 ml, sensitizer 10 ml ultra pure water to 100 ml</td>
<td>100 ml</td>
<td>10 minutes</td>
</tr>
<tr>
<td>First wash</td>
<td>Ethanol 30 ml, ultra pure water to 100 ml</td>
<td>100 ml</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Second wash</td>
<td>Ultra pure water 100 ml</td>
<td>100 ml</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Staining</td>
<td>Stainer 1ml, ultra pure water to 100 ml</td>
<td>100 ml</td>
<td>15 minutes</td>
</tr>
<tr>
<td>Wash</td>
<td>Ultra pure water 100 ml</td>
<td>100 ml</td>
<td>1 minute</td>
</tr>
<tr>
<td>Developing</td>
<td>Developer 10 ml, developer enhancer 1 drop, ultra pure water to 100 ml</td>
<td>100 ml</td>
<td>4-8 minutes</td>
</tr>
<tr>
<td>Stopping</td>
<td>Stopper 10 ml, add directly to developing solution</td>
<td>10 ml</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Final wash</td>
<td>Ultra pure water 100 ml</td>
<td>100 ml</td>
<td>10 minutes</td>
</tr>
</tbody>
</table>

5.3.3.5. Densitometry of SDS-PAGE

- Scan the SDS-PAGE gels digitally (Power LookII; Umax Data Systems, Inc., Industrial Park, Taiwan).
- Measure the band density of $\alpha_1$(I) and $\alpha_2$(I) bands with gel scanning software (1-D Gel Scan; MetaMorph; Universal Imaging Corp., Downingtown, PA).
- Compare the band densities of gels with the respective standard (symatese collagen I) -$\alpha_1$(I) and $\alpha_2$(I) bands densities after adjusting the background.
5.3.4. alamarBlue® assay

- Aspirate the culture medium after the MMC treatment for specific time period.
- Rinse the cell layer briefly using HBSS.
- Make the 10% alamarBlue® reagent in cell culture medium.
- Add 150μL of the cell culture medium containing 10% alamarBlue® reagent in a sample well of 24 well plates.
- Add 150 μL of the cell culture medium and cell culture medium containing 10% alamarBlue® reagent per well without any cell samples as the controls.
- Incubate the samples in a humidified incubator for 4 hours.
- Transfer 100μL of these medium samples into a black 96-well plate.
- Also transfer the 100 μL cell culture medium and cell culture medium containing 10% alamarBlue® reagent as the control in different wells of 96-well plate.
- Read the fluorescence of the media using a microplate reader (Varioskan Flash, Thermo Scientific) at excitation (570nm) and emission (600nm) wavelength respectively.
- Calculate the metabolic activity of the samples using the % reduction of dye, according to the supplier’s protocol and compare with the respective control samples.
5.3.5. Live/Dead® assay

- Aspirate the medium after the MMC treatment for specific time period.
- Rinse the cell layer samples briefly using HBSS.
- Add the calcein-AM and ethidium homodimer solution according to manufacturer's staining protocol (2µM calcein-AM and 4µM EthD-1 in HBSS) (100µl/cm²).
- Incubate the samples in a humidified incubator for 30 minutes.
- Wash the cell layer after this incubation using the HBSS to remove the excess dyes.
- Take at least three images per sample for live as well as dead cells in the same treatment using an Olympus IX81 inverted fluorescence microscope.
- Use the FITC filter and DX red filter for live and dead cells respectively.
- Take at least three samples per treatment for Live/Dead® assay.
- Calculate the percent of live cells after comparing with control cell sample according to the manufacturer’s protocol.
5.3.6. Cell proliferation assay (DNA quantification)

- Discard medium and wash the cell layer using the nuclease free water after culture.
- Add 200 µl of nuclease free water per well (24 well plate).
- Immediately transfer the well plate in -80 °C for freezing for 30 minutes.
- Transfer the well plate at room temperature (25 °C) for thawing.
- Follow two more freeze-thaw cycles as above.
- Transfer the cell samples in pre-labelled cold eppendorf tubes after gentle scratching.
- Centrifuge the samples for five minutes at 1200 RPM.
- Transfer 25µL sample of supernatant into 96-well plate containing 75µL of 1xTE buffer.
- To generate the standard curve dilute the DNA standard as 0, 7.8125, 15.625, 31.25, 62.5, 125, 250 and 500 µg/ml DNA concentrations and pour 100 µL in 3 wells of 96 well plate.
- Add 100 µL of a 1:200 dilution of Quant-iTPicogreen® reagent in each sample.
- Read the plate using a micro-plate reader (Varioskan Flash, Thermo Scientific, Ireland) with an excitation wavelength of 480 nm and an emission wavelength of 525 nm.

5.3.7. Morphology (phase contrast microscopy)

- After specific culture time point take out the samples.
- Discard medium and wash the cell layer using HBSS.
- Take the image using DMLS2 phase contrast microscope at 10X magnification.
5.3.8. Immunocytochemistry/immunofluorescence

- Culture the HCFs cells at 25,000 cells/cm² in a Labtec® chamber plate.
- Do the crowding experiments as above.
- Takeout the culture from the incubator after incubation time (2, 4, 6 days).
- Aspirate the medium from the culture.
- Wash the cellular layer with HBSS (5 minute×3).
- Fix the cellular layer with 2% PFA for 30 minutes.
- Wash the cellular layer with HBSS (5 minute×3).
- Incubate with 3% BSA in 1× HBSS for 30 minutes. Incubate the samples with primary antibody for 1.5 hours at room temperature.

**Primary antibody used**

1. Collagen type I (abcam, ab34719) Rabbit polyclonal to collagen I, 1:200
2. Collagen type III (abcam, ab7778) Rabbit polyclonal to Collagen III, 1:200
3. Collagen type IV (abcam, ab6586) Rabbit polyclonal to Collagen IV, 1:200
4. Collagen type V (abcam, ab7046) Rabbit polyclonal to Collagen V, 1:200
5. Collagen type VI (abcam, ab6588) Rabbit polyclonal to Collagen VI, 1:200
6. Fibronectin (Sigma, F7387) Mouse monoclonal to Fibronectin, 1:200
7. α smooth muscle actin (Sigma, F3777) Mouse to anti-actin 1:400
8. CD34 (Santa Cruz, SC65261) Mouse to CD34, 1:50 dilution
9. Keratocan (MD Bioproducts, 1042008) Mouse to keratocan, 1:50

- Wash the cellular layer with HBSS (5 minutes ×3)
- Incubate the samples with secondary antibody for 30 minutes at room temperature.

**Secondary antibody used**

1. Alexa fluor® 488, chicken anti rabbit (1:400)-Invitrogen
2. Alexa fluor® 488 donkey anti mouse (1:400)-Invitrogen
- Wash the cellular layer with HBSS (5 minutes ×3).
- Fix with 2% PFA for 15 minutes.
- Wash the cellular layer with HBSS (5 minutes ×1).
- Incubate with DAPI (4’,6-diamidino-2-phenylindole) used at 1:4000 dilution. (Invitrogen) for 5 minutes for nuclear staining.
- Wash the cellular layer with DPBS (5 minutes ×3).
- Mount the glass slides with VectaShield (Vector, Ireland) for direct observation.
- Take the images using an Olympus IX81 inverted fluorescence microscope using 10X objective.
Chapter 5

5.3.9. Gelatin zymography

- Culture the HCFs cells at 25,000 cells/cm² in a 24 well plate.
- Do the crowding experiments as above.
- Take out the HCFs cells from the culture at the various time points.
- Aspirate the medium from the culture.
- Collect these medium samples in pre-labelled tube and keep them at -20 °C freezer.
- Take out the sample just before the sample running for zymography gel.
- Mix the sample with equal volume of non-reducing SDS sample buffer (125 mM Tris-HCl [pH 6.8], 20% glycerol, 2% SDS, and 0.002% bromophenol blue).
- Fractionate these samples using SDS-polyacrylamide gel electrophoresis using 10% gels containing 0.1% gelatin.
- Wash the gels briefly with ddH₂O.
- Incubate the gels with 2.5% Triton X-100 solution in distilled water for 30 minutes X2.
- Incubate the gels for 18 hours at 37 °C in a reaction buffer containing 50mM tris pH 7.4, 5mM CaCl₂, 1µM ZnCl₂ to promote recovery of protease activity.
- After this incubation stain the gels with 0.5% Coomassie G250 brilliant blue for 30 minutes.
- Take the images of the gels after de-staining with 30% ethanol/10% acetic acid to get an image with good contrast.
- Compare the relative expression of MMP2 and MMP9.
- Use the uncultured medium with various percentages of new born calf serum or human serum as the controls in respective experiments.
5.3.10. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

5.3.10.1. Extraction of total RNA

Note: Maintain the PCR reaction under laminar air flow to avoid any non host specific DNA.

- After the culture, wash the cell layer briefly using the medium HBSS.
- Add 500 µl of Trizol® per cm² area in cell layer in tissue culture plastic plate.
- Store homogenate for 5 minutes at room temperature (RT) (for nucleoprotein complexes dissociation).
- Add 0.2 ml of chloroform per ml of Trizol® in sterile collecting tube.
- Sake vigorously for 15 seconds by inversion of collecting tubes.
- Incubate for the 15 minutes at room temperature (RT).
- Centrifuge the samples at 12,000 g max (tr/minutes) for 15 minutes at 4°C.
- Following the centrifugation, 3 phases will appear: a lower red phenol-chloroform phase, an inter-phase and an upper aqueous phase (translucent).
- Take out the clear upper aqueous phase and add it in a fresh pre labelled tube.
- Add equal volume of 70% ethanol (in 3 equal aliquots) and mix gently by inversion.
- Apply 700 µl samples from above mixture to RNeasy column, centrifuge for 15 seconds at 8,000g and discard flow-through. Repeat this process for the remaining sample if any.
- Add 350 µl of RW1 buffer to centre of column, centrifuge for 15 seconds at 8,000g, discard flow-through.
- Add 10 µl DNAse stock solution with 70 µl RDD buffer and add the DNAse incubation mix directly onto the RNeasy column.
- Incubate it at room temperature for 15 minutes.
- Add 350 µl of RW1 buffer to centre of column, centrifuge for 15 seconds at 8,000g, discard flow-thorough.
- Transfer column to new 2ml collection tube. Add 500 µl RPE buffer to the centre of column, centrifuge for 15 seconds at 8,000 g, and discard flow-through.
Add 500μl of RPE buffer to centre of column, centrifuge for 15 seconds at 8,000g, discard flow-thorough, centrifuge for a further 2 minutes at 8,000g.

Transfer column to new 1.5 ml tube, add 30μl RNAse-free water onto the column, incubate at RT for 1 minute, centrifuge for 1 minute at 8,000g.

Take back this 30μl of elute and add again onto the column, incubate at room temperature for 1 minutes, centrifuge for 1 minute at 8,000g.

Split the elutes in 3 parts.

Determine the concentration of RNA using the Nanodrop and freeze the samples at -80°C.

Determine of RNA quantity and purity.

Dilute the RNA at 1:50 dilution with RNAse free water (if need).

Calibrate the Nanodrop using the RNAse free water.

Measure the absorbance at 260 nm.

1 unit of $A_{260} = 40 \, \mu g/ml$ of RNA

Concentration of RNA sample = 40 * $A_{260}$ * dilution factor = $x \, \mu g/ml$

Quantity of RNA = concentration * volume of sample in ml = $x \, \mu g$

Purity of RNA

The purity of RNA is evaluated using the ratio of absorption values at 260 & 280 nm

If the ratio $A_{260}/A_{280}$ value is superior at 1.8-1.9 the RNA is of good quality (max of the ratio 2.2).

The ratio of reading at $A_{260}/A_{280}$ provides an estimate of the purity of RNA with respect to the contaminants that absorb in the UV, such as protein. It’s influenced by the pH. Since water is not buffered, the pH and the resulting $A_{260}/A_{280}$ ratio can vary greatly. But the extinction coefficient is calculated in water, so for the concentration it’s better to calculate in water.

5.3.10.2. Reverse transcription (RT)

Take sterile, pre labelled and nuclease-free, tubes, pre-chilled on ice.

For 20μl reverse transcription reaction add 1μl of RNA sample (water in case of control), 1μl of random primers and 3 μl of nuclease free water in the tubes.
• Incubate the tubes at 70 °C for 5 minutes (denature the target and the primers)
• Quick-chill the tube at 4 °C for 5 minutes and hold on ice (or direct in the ice bath)
• Keep these tubes on ice and prepare the reverse transcription mix.

**Preparation of reverse transcription mix**

• Prepare reverse transcription mix according to the following ratio

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl) per 1RT</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease free water</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
<td>ImPromo-II TM 5X reaction buffer</td>
<td>4</td>
<td>1X</td>
</tr>
<tr>
<td>MgCl₂ (25 mM)</td>
<td>2.4</td>
<td>3mM</td>
</tr>
<tr>
<td>dNTP mix (10mM each dNTP)</td>
<td>1</td>
<td>0.5mM</td>
</tr>
<tr>
<td>Recombinant RNaSin ribonuclease inhibitor (20/40µ per µl)</td>
<td>1</td>
<td>1µ / µl</td>
</tr>
<tr>
<td>ImPromo-II TM reverse transcriptase</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Final volume RT mix per 20 µl reaction</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

**Note: Begin with the biggest volume first**

• Add the reverse transcriptase in last
• Keep mix and products all the manipulation on ice before incubation
• Vortex gently to mix
• Add 15µl of the reverse transcription mix in 5µl of already prepared RNA template as above.

**Start the reverse transcription as following**

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annealing</td>
<td>25 °C</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Extension</td>
<td>42 °C</td>
<td>60 minutes</td>
</tr>
<tr>
<td>Heat-inactivation reverse transcription</td>
<td>70 °C</td>
<td>15 minutes</td>
</tr>
</tbody>
</table>

• After, synthesis of cDNA, proceed for PCR or store frozen.
5.3.10.3.1 Preparation

- Use sterile, pre labelled nuclease-free, tubes, pre chilled on ice.
- Prepare a mix for all PCR according to following formulae.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per PCR</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl₂ (25 mM)</td>
<td>3</td>
<td>1.5mM</td>
</tr>
<tr>
<td>Reaction buffer (5X)</td>
<td>10</td>
<td>1X</td>
</tr>
<tr>
<td>Nucleotide mix (10mM)</td>
<td>1</td>
<td>200µM</td>
</tr>
<tr>
<td>Forward pmol</td>
<td>1</td>
<td>0.1-1 µM</td>
</tr>
<tr>
<td>Reverse pmol</td>
<td>1</td>
<td>0.1-1 µM</td>
</tr>
<tr>
<td>Taq polymerase 5µl/ µl</td>
<td>0.25</td>
<td>1.25 µ/ 50 µl</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1</td>
<td>&lt; 0.5 µg/50 µl</td>
</tr>
<tr>
<td>Qsp nuclease free water</td>
<td>32.75</td>
<td></td>
</tr>
<tr>
<td>Final volume</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

Note: Begin by the biggest volume and vortex MgCl₂ before use.

- Add the Taq polymerase in last.
- Keep mix and products all the manipulation on ice (important do not let the Taq polymerase at room temperature!!!). The list of designed primers is given in table 2.1 / 3.1.
- Vortex the mixture before the distribution in tubes.
- Centrifuge the tubes a little if it’s necessary.
- Disturb the mix in the tube.
- Add cDNA template in function of the concentration.
- Start the PCR program on PCR Engine.
5.3.10.3.2. Optimisation of designed primers for each genes using agarose gel electrophoresis

5.3.10.3.2.1. Materials/reagents required

- Agarose, 6X sample Buffer
- TAE buffer (4.84 g Tris Base, 1.14 ml of Glacial acetic acid, 2 ml of 0.5M EDTA (pH 8.0), bring the total volume up to 1L with distilled water)
- Power supply, staining tray, pipette and tips, gloves
- DNA ladder standard
- Electrophoresis chamber, gel casting tray and combs
- SYBR Safe DNA gel stain

5.3.10.3.2.2. Preparing agarose gel

- Weigh 1g of agarose powder and add it to 500 conical flask
- Add 100ml of TAE buffer to the flask (depending on number of samples and size of casting tray available, total volume of the gel may vary)
- Melt the agarose until the solution become clear. It may take around 1 minute.
- Do not over boil the solution as it may boil out the flask.
- Let the solution cool to about 50-55 °C by swirling occasionally for even cooling.
- Add 10µl of SYBR safe DNA gel stain to the solution and mix by swirling.
- Place appropriate comb in the casting tray at appropriate position.
- Pour the agarose solution in the gel casting tray. Make sure that the ends of the tray are sealed properly.
- Allow the gel to cast in the tray until it is solid. It should take around 30 minutes.
- Pull out the comb carefully.
- Place the gel in the electrophoresis chamber and add enough TAE buffer so that the level of the buffer is at least 2-3 mm over the gel.
5.3.10.3.2.3. Loading the gels

- Note down the order in which samples will be loaded in the gel.
- On a paraffin film, add 8 µl of each DNA sample one beside the other in the sequence the samples will be loaded. Use separate tip each time. Add 2 µl of loading dye to each sample.
- Using a fresh loading tip, each sample with the loading dye and load the sample in the appropriate well.
- Pipette 10 µl of the DNA ladder standard in at least one of the well.
- If there are empty wells load them with loading dye appropriately diluted in TAE buffer.

5.3.10.3.2.4. Running the gels

- Place the lid on the gel box and connect the electrode correctly (red to positive and black to negative electrode).
- Turn on the power supply and set the voltage to 100 volts.
- Check to make sure that current is running thorough the buffer. This can be confirmed by presence of bubbles forming on each electrode.
- Check that the direction of current is correct. This can be checked by observing the direction of the movement of blue loading dye after about couple of minutes of running the gels.
- Let the gel run until the blue dye reaches the end of gel. This generally takes 40-45 minutes.
- Turn off the power supply and disconnect all the wires.
- Remove the lid of electrophoresis chamber and using gloves carefully remove the gel with the tray.

5.3.10.3.2.5. Observing the gels

- Place the gel on to the trans-illuminator and ensure the safety door of the dark room is shut down securely.
- Select SBYR safe filter. The trans-illuminator should turn on automatically.
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- Observe the image of gel on the computer screen. The image may be adjusted by opening or closing the picture or by increasing or decreasing the exposure time.
- Once satisfied with adjustment click capture to capture image. At this stage changes can be made such as cropping the image for new area of interest or enhance by adjusting the brightness/contrast etc. Save the image.
- Switch off the trans-illuminator remove the gel from the trans-illuminator, wrap in a tissue paper, discard in appropriate waste bin and wipe dry the trans-illuminator (Figure 5.2).

Figure 5.2. | Agarose gel electrophoresis of the reverse transcribed DNA.

5.3.10.4. Evaluation of primers

- Do the reverse transcription to prepare the cDNA as described above.
- Add 2µl of cDNA prepared using designed primers at various dilutions (1000, 200, 40, 8 & 1.6) + 18 µl of SYBR® Green mix for each sample in 96 well plate.
- Run the qPCR (Steponeplus™) and calculate the relative thresholds (Ct) and evaluate the R² values for various primers pairs.

Note: If the R² value is greater than 0.85, the primers can be used for experiments (Figure 5.3).
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5.3.10.5. Quantitative polymerase chain reaction (qPCR)

- Do the reverse transcription to prepare the cDNA as described above.
- Add 2μl of cDNA and 18 μl of SYBR® Green mix for each sample in the well of 96 well plate.
- Take the control in the same way avoiding the cDNA and adding the nuclease free water and 18μl of SYBR® Green mix.
- Take the 18s primer samples as control for each sample.
- Centrifuge the 96 well plate briefly at 4 °C
- Avoid the light exposure to the SYBR® Green mix.
- Insert the 96 well plate in step one plus instrument and start the PCR reaction.
- Calculate the relative thresholds (Ct) and total DNA using the formula and normalised the samples using the 18s primers.
- Calculate the relative gene expression of interest using the $2^{-\Delta\Delta Ct}$ Method.

**Machine protocol (PCR)**

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95 °C</td>
<td>2 minutes</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>1 minute</td>
<td>25</td>
</tr>
<tr>
<td>Annealing</td>
<td>56 °C</td>
<td>30 seconds</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72 °C</td>
<td>1.5 minutes</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72 °C</td>
<td>5 minutes</td>
<td>1</td>
</tr>
<tr>
<td>Soak</td>
<td>4 °C</td>
<td>indefinite</td>
<td>1</td>
</tr>
</tbody>
</table>
Figure 5.3. Efficiency of the designed primers in qRT-PCR.
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5.3.11. Development of cell sheets

- Mix the 65 % N-isopropylacrylamide / 35 % N-tert-butylacrylamide (NTBA) copolymer [poly(NIPAAM-N-t-BAM)] at the ratio of 40 µg/ml in alcohol.
- Leave the polymer solution to mix overnight with continuous shaking.
- Add the equal volume of copolymers solution to the poly(L-lysine) solution at 1:1 volume ratio.
- Allow to shake the copolymer and poly(L-lysine) overnight with continuous stirring again.
- Pour ~500 µl of this polymer solution to the petri-dish (diameter - 3.5 cm)
- Spread the copolymer uniformly on this dish surface.
- Leave these petri dishes in to the desiccators to dry out the excess of polymer solution.
- Leave the petri dishes in to the vacuum oven at 60 °C overnight.
- Keep the petri dishes under the ultraviolet light for 2 hours for the sterilisation.
- Seed the HCFs cells at 25,000 cells/ cm² in keratocyte medium.
- Change the medium after 24 hours of cell seeding with keratocyte medium containing 0.5% human serum and 75µg/ml of CR or FC combination (Ficoll®70 + Ficoll®400: 37.5mg/ml + 25mg/ml) and l ascorbic acid.
- Incubate the culture for next 4 or 6 days in a humidified chamber at 37 °C.
- After culture time point take out the petri dishes from the incubator and leave it at 10 °C for 30 - 45 minutes.
- Collect the HCFs cell seed with gentle scrapping in corners without breaking it.
5.3.12. Atomic force microscopy (AFM)

- Seed the human corneal fibroblast on 4-well Lab-Tek™ II chamber slides at 25,000 cells/cm².
- Incubate the cells in 95% air and 5% CO₂ in humidified chamber.
- Change the medium after 24 hours of cell seeding with optimum amount of crowder molecules and l ascorbic acid.
- After specific culture time point, wash the cell layers with HBSS.
- Fix the cell layers with 3% paraformaldehyde at room temperature for 15 minutes.
- Wash the cell layer three times with HBSS.
- Dehydrate the samples serially using 30%, 50%, 70%, 90% and 100% ethanol.
- Perform the AFM (MFP-3D, Asylum Research) using rectangular Si cantilevers (SSS-NCH, Nanosensors) each having a nominal resonance frequency of 330 kHz and a spring constant of 42 N/m.
- Record the AFM images using amplitude modulation mode in an ambient environment after drying the sample with nitrogen gas.
5.3.13. Masson’s trichrome staining

- Fix the cell sheets in Bouin’s solution for 1 hour at 56 °C after paraformaldehyde fixation.
- Incubate the samples in Weigert's iron haematoxylin staining solution for 10 minutes, followed by brief rinsing with running tap water.
- Wash the samples with distilled water.
- Incubate the samples with Biebrich scarlet-acid fuchsin solution for 10 - 15 minutes and wash again with the distilled water.
- Incubate the samples with phosphomolybdic-phosphotungstic acid solution for 15 minutes for differentiation.
- Transfer the samples directly in aniline blue solution and stained for 10 minutes.
- Rinse it briefly using distilled water and differentiated again using 1% acetic acid solution for 5 minutes.
- Dehydrate the samples quickly thorough 95% ethyl alcohol, absolute ethyl alcohol (these steps wipe off Biebrich scarlet-acid fuchsin staining) and cleared in xylene after brief washing in distilled water.
- Finally, take the images using the microscope at 100X after mounting with resinous mounting medium (BX51 microscope).
5.3.14. Picro-sirius red staining

- Seed the human corneal fibroblast on 4-well Lab-Tek™ II chamber slides at 25,000 cells/cm².

- Incubate the cells in 95% air and 5% CO₂ in humidified chamber.

- Change the medium after 24 hours of cell seeding with optimum amount of crowder molecules and l-ascorbic acid.

- After specific culture time point of culture, wash the cell layers with HBSS.

- Fix the cell layers with 3% paraformaldehyde at room temperature for 15 minutes.

- Keep the paraformaldehyde fixed cell sheets under running tap water for short time.

- Incubate the cell sheets with Weigert’s haematoxylin solution for 10 minutes.

- Rinse it under running tap water for brief washing.

- Stain with 0.2% phosphomolybdic acid hydrate.

- Rinse it under running tap water again for brief time (5 minutes).

- Stain the cell-sheets with Picro-sirius red followed by washing in acidified water.

- Dehydrate the samples through a series of ethanol baths (70%, 80%, 90%, and finally 100%).

- Perform the final dehydration in xylene for at least 5 minutes.

- Mount the samples on slides using DPX mounting media.

- Capture the images using an Olympus BX-51 microscope.
5.3.15. van-Gieson staining

- Wash the cell layer using DPBS (pH 7.4) after time point (4 days in case of CR and 6 days in case of FC).
- Fix the cell layers using 3.0% paraformaldehyde for 30 minutes at room temperature.
- Wash the samples in distilled water briefly.
- Incubate the samples in Weigert’s working hematoxylin solution for 10 minutes (equal parts of Weigert’siron hematoxylin A and Weigert’siron hematoxylin B).
- Wash in distilled water, briefly.
- Stain the samples for 1 - 3 minutes in van Gieson’s solution.
- Wash is distilled water briefly.
- Dehydrate the samples in 95% alcohol and absolute alcohol (2 changes each for 5minutes).
- Clear the samples using the xylene (5 minutes X2).
- Mount the samples using the paramount.
- Take the images using BX 51 Olympus microscope.
5.3.16. Light transmittance

- Aspirate the culture medium after crowding experiment at 4 and 6 days (CR and FC respectively).
- Wash the cell layer briefly, with 100$\mu$l HBSS.
- Add 100$\mu$l HBSS in each sample well after HBSS washing.
- Add the HBSS only in black wells without any cell sheet as the control.
- Take wells without any HBSS/cells and black dye as control for zero absorbance and 100% absorbance.
- This black dye should show approximately 100% absorbance, i.e. 0% transmittance,
- Take the absorbance value 0.0 for wells without any cells or HBSS (100% transmittance as a reference).
- Measure the optical density of the samples using the spectrophotometer (Varioskan Flash, Thermo Scientific) between the 380-780 nm wavelength with a resolution wavelength of 5nm.
- Calculate the percentage transmission using the formula below.

\[
\text{% Transmittance} = 100(10^{-A})
\]

where A is absorbance measured.
5.3.17. Proteomic analysis

5.3.17.1. Protein extraction - Qproteome™ mammalian protein preparation kit (Qiagen, UK)

- Wash the cell layers twice after crowding experiment with HBSS.
- Scrape the cell layer gently using a cell scraper in the presence of ice cold HBSS.
- Transfer the scrapped cell layer suspension to the pre-chilled 1.5ml tubes.
- Centrifuge this solution at 450g for five minutes at 4 °C.
- Lyse the cell layer gently with cell lysis buffer containing Benzonase® nuclease and protease inhibitor after discarding the supernatant.
- Centrifuge the cell suspension from previous step again at 14,000g at 4 °C for 10 minutes,
- Agitate it on a rotary shaker for 5 minutes.
- Transfer the supernatant after centrifugation, into pre-chilled 0.5ml tubes (Protein LoBind Tubes, UK) and freeze-dried.
- Freeze dry the samples to make the cell pellet.

5.3.17.2. Mass spectroscopy

**General.** High-thouroughput proteomic profiling was performed at the mass spectrometry core facility of the Beth Israel Deaconess Medical Center (BIDMC) using the Applied Biosystems (ABI, Foster City, CA) 4-plex iTRAQ labelling kit with the 4700 MALDI TOF/TOF system.

**Steps:**
- Re-suspend the protein pellets from each sample using a protein dissolution buffer (10 mM triethyl ammonium bicarbonate (TEAB), pH 8.5).
- Assign the protein sample to one of four isobaric tags as per the manufacturer’s instructions.
- Pool the tagged samples together and subjected to mass spectrometry analysis.
- Fractionate the samples using strong cation exchange (SCX).
- Separate the samples by reverse phase chromatography using a Dionex™ UltiMate 3000 RSLCnano system.
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- Spot the eluted protein fractions after reverse phase chromatography, onto a MALDI (matrix-assisted laser desorption ionisation) plate (ABI 4800 OptiTOF).
- Mix the spotted plates with α-Cyano-4-hydroxycinnamic acid (CHCA) ionisation matrix (Sigma Aldrich) at a ratio of 1:2 using a Probot printing robot (Dionex, Sunnyvale, CA).
- Perform the mass spectrometry analysis of the spotted plate using an ABI 4800 Plus MALDI-TOF/TOF (time-of-flight/time-of-flight) tandem MS system.

5.3.17.3. Analysis

**General.** The time-of-flight of the protein is proportional to the molecular weight. The final read-out graph is a multi peak spectrum with different peak intensity which corresponds to the relative protein amount and the peak location corresponds to the precise protein or peptide molecular weight.

**Steps:**

- Perform the data analysis using Protein Pilot 2.0 software (ABI, Foster City, CA) on SWISS-PROT, TrEMBL (www.ebi.ac.uk/swissprot) and NCBI (www.ncbi.nlm.nih.gov/) non-redundant protein databases.
- Keep the unused score at more than 1.3 to get the confidence interval more then 90, and compare the relative peptide value (at 95%) between the samples.
- Validate the selected ECM proteins detected in proteomics results using immunocytochemistry and measurement of fluorescent intensity using Olympus IX81 inverted microscope.
5.4. Proteomic analysis of HCFs using negative charged macromolecules

Table 5.3. | Proteomic analysis of HCFs using negative charged macromolecules.

Table 5.3.1. | No MMC Vs DxS (proteomic analysis).

Mass spectrometry results of various proteins from human corneal fibroblast in the presence of 0.5% HS under crowding condition with DxS 100µg/ml. ND indicates Not Detected.

5.3.1.1. | Collagen and collagen related proteins in cell layer.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Proteins</th>
<th>Accession</th>
<th>Control</th>
<th>DxS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Collagen alpha-1(I) chain OS=Homo sapiens GN=COL1A1 PE=1 SV=5</td>
<td>sp</td>
<td>P02452</td>
<td>CO1A1_HUMAN</td>
</tr>
<tr>
<td>2</td>
<td>Uncharacterized protein OS=Homo sapiens GN=COL6A3 PE=4 SV=1</td>
<td>tr</td>
<td>E9PFQ6</td>
<td>E9PFQ6_HUMAN</td>
</tr>
<tr>
<td>3</td>
<td>Uncharacterized protein OS=Homo sapiens GN=COL1A2 PE=4 SV=1</td>
<td>tr</td>
<td>F5H299</td>
<td>F5H299_HUMAN</td>
</tr>
</tbody>
</table>

5.3.1.2. | Fibronectin in cell layer.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Proteins</th>
<th>Accession</th>
<th>Control</th>
<th>DxS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Uncharacterized protein OS=Homo sapiens GN=FN1 PE=4 SV=1</td>
<td>tr</td>
<td>F8W7G7</td>
<td>F8W7G7_HUMAN</td>
</tr>
</tbody>
</table>

5.3.1.3. | Laminin in cell layer.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Proteins</th>
<th>Accession</th>
<th>Control</th>
<th>DxS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Prelamin-A/C OS=Homo sapiens GN=LMNA PE=1 SV=1</td>
<td>sp</td>
<td>P02545</td>
<td>LMNA_HUMAN</td>
</tr>
</tbody>
</table>
### 4.3.1.4. Tubulins in cell layer.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Proteins</th>
<th>Accession</th>
<th>Control</th>
<th>DxS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tubulin, alpha 3, isoform CRA_c OS=Homo sapiens GN=TUBA1A PE=3 SV=1</td>
<td>tr</td>
<td>G3V1U9</td>
<td>G3V1U9_HUMAN</td>
</tr>
<tr>
<td>2</td>
<td>Tubulin beta polypeptide OS=Homo sapiens GN=TUBB PE=3 SV=1</td>
<td>tr</td>
<td>Q5JP53</td>
<td>Q5JP53_HUMAN</td>
</tr>
</tbody>
</table>

### 5.3.1.5. Other ECM and cytoskeleton proteins.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Proteins</th>
<th>Accession</th>
<th>Control</th>
<th>DxS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6</td>
<td>sp</td>
<td>P04264</td>
<td>K2C1_HUMAN</td>
</tr>
<tr>
<td>2</td>
<td>Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3</td>
<td>sp</td>
<td>P35527</td>
<td>K1C9_HUMAN</td>
</tr>
<tr>
<td>3</td>
<td>Keratin, type I cytoskeletal 16 OS=Homo sapiens GN=KRT16 PE=1 SV=4</td>
<td>sp</td>
<td>P08779</td>
<td>K1C16_HUMAN</td>
</tr>
<tr>
<td>4</td>
<td>Cytoskeleton-associated protein 4 OS=Homo sapiens GN=CKAP4 PE=1 SV=2</td>
<td>sp</td>
<td>Q07065</td>
<td>CKAP4_HUMAN</td>
</tr>
<tr>
<td>5</td>
<td>Myosin-9 OS=Homo sapiens GN=MYH9 PE=1 SV=4</td>
<td>sp</td>
<td>P35579</td>
<td>MYH9_HUMAN</td>
</tr>
<tr>
<td>6</td>
<td>Vimentin OS=Homo sapiens GN=VIM PE=1 SV=4</td>
<td>sp</td>
<td>P08670</td>
<td>VIME_HUMAN</td>
</tr>
<tr>
<td>7</td>
<td>Annexin A2 OS=Homo sapiens GN=ANXA2 PE=1 SV=2</td>
<td>sp</td>
<td>P07355</td>
<td>ANXA2_HUMAN</td>
</tr>
</tbody>
</table>
5.3.1.6. | Enzymes in cell layer.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Proteins</th>
<th>Accession</th>
<th>Control</th>
<th>DxS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Proto-oncogene serine/threonine-protein kinase mos OS=Homo sapiens GN=MOS PE=2 SV=1</td>
<td>sp</td>
<td>P00540</td>
<td>MOS_HUMAN</td>
</tr>
</tbody>
</table>

5.3.1.7. | Matrix metalloproteinases (MMPs) in cell layer.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Proteins</th>
<th>Accession</th>
<th>Control</th>
<th>DxS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alpha-2-macroglobulin OS=Homo sapiens GN=A2M PE=1 SV=3</td>
<td>sp</td>
<td>P01023</td>
<td>A2MG_HUMAN</td>
</tr>
</tbody>
</table>
Table 5.3.2. | No MMC Vs CR (proteomic analysis).
Mass spectrometry results of various proteins from human corneal fibroblast in the presence of 0.5% HS under crowding condition with CR 75µg/ml. ND indicate Not Detected.

5.3.2.1. | Collagen and collagen related proteins in cell layer.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Proteins</th>
<th>Accession</th>
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<td>Isoform 1 of Collagen alpha-1(II) chain OS=Homo sapiens GN=COL2A1</td>
<td>sp</td>
<td>P02458-1</td>
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<td>E9PFQ6</td>
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5.3.2.2. | Tubulins in cell layer.

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<tr>
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<td>Tubulin, alpha 3, isoform CRA_c OS=Homo sapiens GN=TUBA1A PE=3 SV=1</td>
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<td>G3V1U9</td>
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### 5.3.2.3. | Other ECM and cytoskeleton proteins.

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<td>Myosin-9 OS=Homo sapiens GN=MYH9 PE=1 SV=4</td>
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<td>Annexin A2 OS=Homo sapiens GN=ANXA2 PE=1 SV=2</td>
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<td>4</td>
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### 5.3.2.4. | Enzymes in cell layer.

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5.5. Research outputs

5.5.1. Award

Best Poster presentation award at 1st Matrix Biology Ireland conference (MBI 2014) at National University of Ireland Galway, Ireland

5.5.2. Publications


5.5.3. Conference presentations


Chapter 5


Chapter 5

Society For Biomaterials 2014 Annual Meeting & Exposition, Denver, CO, USA, 16th – 19th April, 2014.
Chapter 5


