Review

Nanoscale Topographies for Corneal Endothelial Regeneration

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Abstract: The corneal endothelium is the innermost layer of the cornea that selectively pumps ions and metabolites and regulates the hydration level of the cornea, ensuring its transparency. Trauma or disease affecting human corneal endothelial cells (hCECs) can result in major imbalances of such transport activity with consequent deterioration or loss of vision. Since tissue transplantation from deceased donors is only available to a fraction of patients worldwide, alternative solutions are urgently needed. Cell therapy approaches, in particular by attempting to expand primary culture of hCECs in vitro, aim to tackle this issue. However, existing cell culture protocols result in limited expansion of this cell type. Recent studies in this field have shown that topographical features with specific dimensions and shapes could improve the efficacy of hCEC expansion. Therefore, potential solutions to overcome the limitation of the conventional culture of hCECs may include recreating nanometer scale topographies (nanotopographies) that mimic essential biophysical cues present in their native environment. In this review, we summarize the current knowledge and understanding of the effect of substrate topographies on the response of hCECs. Moreover, we also review the latest developments for the nanofabrication of such bio-instructive cell substrates.

Keywords: substrate topography; corneal endothelial cells; nanofabrication; cornea; corneal cell culture; topography; regenerative medicine; advanced cell culture substrates

1. Introduction

The cornea is a transparent tissue located in the anterior segment of the eye. This avascular tissue allows light to enter the eye and accounts for most of its refractive power [1]. It is composed of five layers (Figure 1): the epithelium, the Bowman's layer, the stroma, the Descemet's membrane and the endothelium. The corneal epithelium is the outermost layer and consists of a stratified sheet of epithelial cells residing on the Bowman's layer. The corneal stroma accounts for 80 to 90% of the total tissue volume and provides the cornea with mechanical strength. This layer is composed of highly structured collagen fibers and other extracellular matrix (ECM) proteins produced by the corneal keratocytes, which maintain the stromal homeostasis. The inner part of the cornea is composed of a monolayer of hexagonal endothelial cells that is in contact with the stroma through the Descemet's membrane (Figures 1 and 2), the latter being characterized by well-defined architecture, porosity and collagen fibrils dimension [2,3]. It is believed that a dual relationship exists between the Descemet's membrane and the corneal endothelium, where the pathophysiology of both of these parts can equally affect or be affected by the state of the other [3].

These (human) corneal endothelial cells (hCECs) play a crucial role in maintaining the corneal transparency by actively pumping ions and other metabolites from the stroma to the aqueous humor of the eye, consequently regulating the corneal hydration level. Unlike vascular endothelial cells, hCECs derive from the neural crest during development. In adults, hCECs are retained in a non-dividing G1 phase [4] and, thus, cannot contribute to the regeneration of this layer through cell division [5]. Corneal dystrophies, damage after surgery or infections can cause a loss of hCECs. The pathological profile of corneal endothelial disease is characterized by cornea edema and opacification. Between 2012 and 2013, about 200,000 corneal transplantations were performed worldwide, with an estimation of 12.7 million patients awaiting transplantation [6].
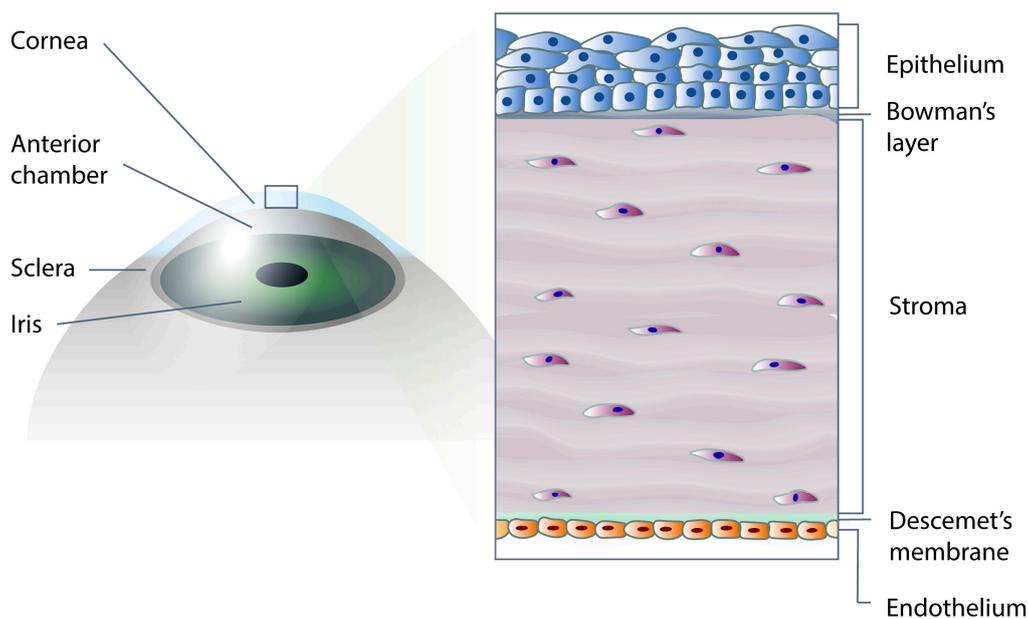

Figure 1. Anatomy of the cornea with schematic representation of its five main layers. Epithelial cells are indicated in blue, stromal keratocytes in purple and endothelial cells in orange.
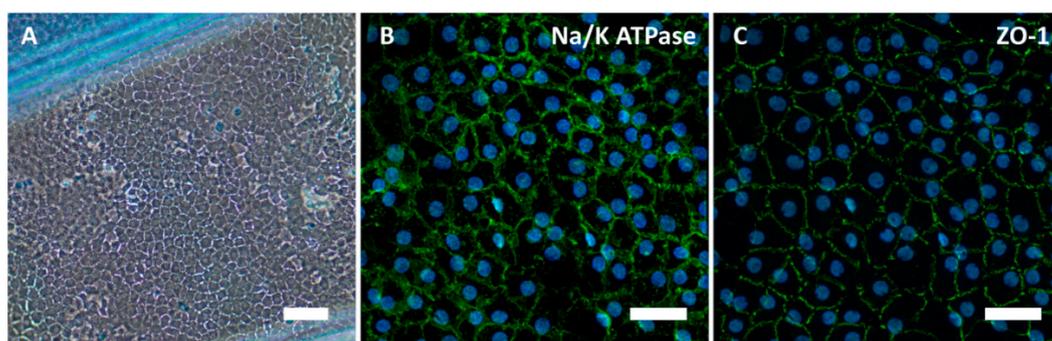

Figure 2. Human corneal endothelium biopsy analysis. Brightfield image of the typical hexagonal morphological pattern exhibited by human corneal endothelial cells (hCECs) (**A**); immunofluorescence analysis of native human corneal endothelium (**B** and **C**); cell nuclei were stained with Hoechst and are shown in blue. Functional marker Na^+/K^+-ATPase (**B**) and tight junction marker ZO-1 (**C**) are shown in green. Scale bars: (**A**): 100 μm; (**B**) and (**C**): 50 μm.

Currently, the state-of-the-art therapy for corneal endothelial disease involves the selective replacement of the corneal endothelium with that of a donor [7]. However, only one donor cornea is available for every seventy patients in need [6]. The primary culture of hCECs is a promising alternative cell source to treat corneal endothelial disease [8], which would challenge the current one donor–one patient paradigm to make the therapy available for more patients. Despite hCECs being arrested in a non-proliferative state, it is

possible to force their in vitro proliferation by culturing them with different basal media compositions, growth factors, such as fibroblast growth factor 2 or nerve growth factor, and other additives, such as ascorbic acid or calcium chloride [9]. Nonetheless, forcing hCECs to exit their quiescent state to enter a proliferative state by solely using biochemical factors is known to cause an undesired endothelial-to-mesenchymal transition resulting in a loss of function [10]. Despite the current efforts to optimize the primary expansion of hCECs, these cells tend to undergo phenotypical alterations already after two passages [11–13], suggesting that a longer culture period in a non-physiological environment leads to seemingly irreversible changes in cell behavior, therefore limiting the number of cells that can be derived from a donor cornea. Moreover, most successful cultures are from donors aged less than 40 years. Current standards for evaluating hCECs in vitro culture consist of assessing cell morphology and a panel of markers, such as Na^+/K^+-ATPase (an ion transporter assessed as functionality marker, Figure 2B), zona occludens-1 (ZO-1) (a tight junction protein, Figure 2C) and CD166 (a transmembrane protein ligand of CD6), none of which can be considered highly specific when analyzed independently. As an alternative to primary culture of hCECs, scientists have explored the differentiation of pluripotent stem cells to hCECs. Nevertheless, such differentiation protocols are still at an early developmental stage and need further improvements to prove that the genotype, phenotype and functionality of the differentiated cells resembles that of native hCECs [14–16]. Overall, the challenges associated with the primary culture of hCECs remain a barrier to their therapeutic application. Thus, there is a need for more advanced approaches that perhaps combine biophysical and biochemical factors in order to better control hCECs' phenotype over a prolonged period of time. Novel bioengineering tools have the potential to generate more defined and more physiological cell culture conditions also for hCECs.

To support the expansion and differentiation of cells in vitro, advanced, bioengineered cell culture substrates have been increasingly employed as a means to mimic biophysical cues that are originally present in native ECM environments [17–19]. These can be obtained by altering substrate features, such as stiffness, wettability, surface energy and topography through suitable fabrication and surface modification processes.

Micrometer scale and nanometer scale topographies' (microtopographies and nanotopographies') structures on cell culture surfaces have been shown to modulate cell behavior, for example by increasing cell adhesion, proliferation, migration, survival, differentiation and phenotype maintenance in many applications [17,19–22]. The addition of instructive topographies on cell culture scaffolds could, therefore, provide significant advantages compared to conventional flat (smooth) 2D substrates, as the first can offer biophysical cues that more closely replicate those present in the native ECM. Surface topographies have already been shown to modulate mesenchymal-to-endothelial/epithelial transition [23–26] and vice versa [27–30] in several others applications. However, research focusing on the interaction between hCECs and surface topography of the culture substrate remains relatively less explored compared to other (corneal) cell types, despite the fact that growing evidence (as reported in the next sections as well as from preliminary work by our group) is showing that subcellular topographies can also have a significant impact on hCECs' expansion and differentiation. In addition, although systematic data on how a defined microenvironment can be used to better guide corneal endothelial cells are essential to design/improve future studies, these are not yet available.

In this review, we briefly describe the range of nanofabrication techniques that can potentially be used to create well-defined cellular or subcellular topographic features for the culture of primary hCECs. We also summarize current knowledge and understanding of the effect of substrate topography on hCEC responses based on the current literature. This information will provide a comprehensive and concise overview of the current state-of-the-art in bio-instructive nanotopographies for hCECs that will help the readers to design their own studies in the field of corneal endothelial regenerative medicine.

2. Nanofabrication Techniques to Design Instructive Cell Substrates

A systematic study on the effect of subcellular topographies was so far difficult to realize also due to the complexity and high costs to produce large-scale nanopatterned areas for extensive cell studies. However, to gain a better understanding of the tools available to conduct such assays with hCECs, a methodic description of the corresponding nanofabrication techniques is essential.

Some of the current advanced nanofabrication techniques that have demonstrated to generate instructive cell substrates [31] are shown in Figure 3. Recapitulating key architectural features of the Descemet's membrane could be one of the most effective approaches for controlling hCECs expansion and phenotype in vitro. Nevertheless, fabricating multiscale and hierarchical structures with a precise control over porosity, fiber diameter and the overlaying hexagonal honeycomb feature, as in in vivo corneas, is challenging to achieve by a single technique and would likely require a combination of multiple nanofabrication techniques, some of which have not been widely employed in this field.
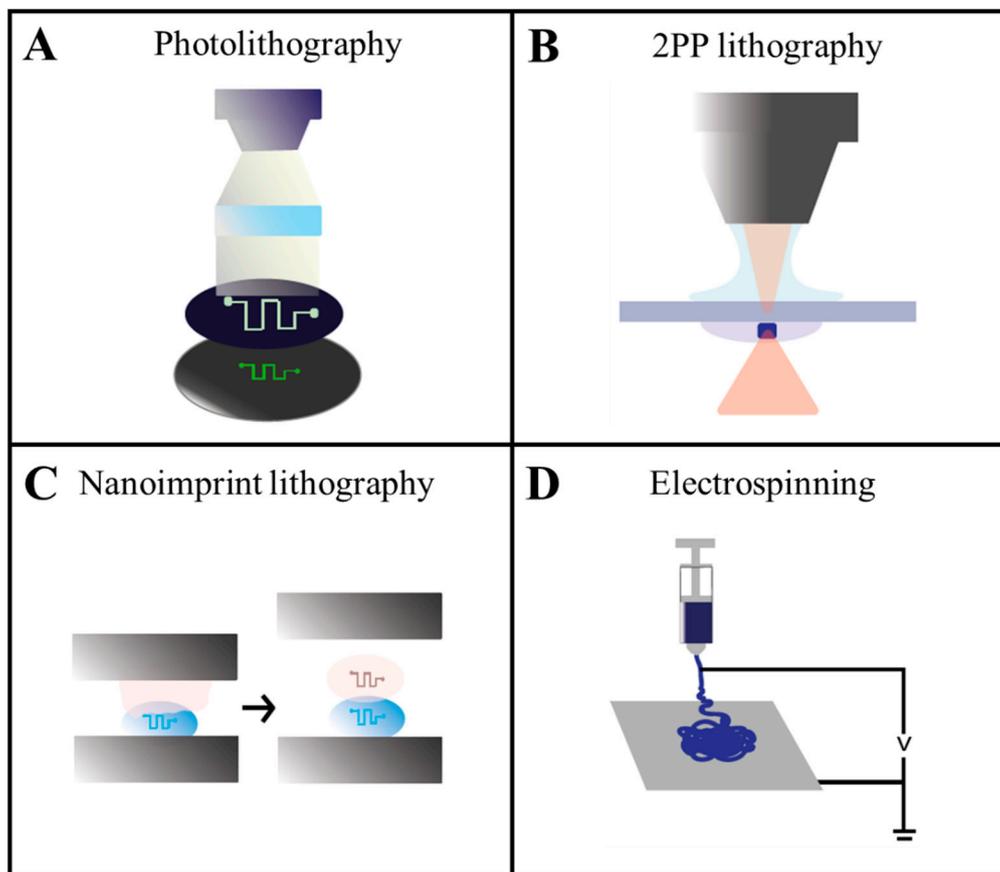

Figure 3. Examples of advanced fabrication tools that can be used for producing bio-instructive substrates for the culture of hCECs. (**A**) Photolithography for transferring a pattern into a photosensitive material. A photoresist is selectively exposed to UV light through a partially light-blocking photomask placed between the UV source and the resist; (**B**) 3D direct laser writing technique based on two-photon polymerization (2PP) lithography. This technique can generate 3D structures from photosensitive materials with resolutions <100 nm; (**C**) nanoimprint lithography. Surface features are transferred onto a polymer material through a mold (stamp) by means of heat or UV light; (**D**) electrospinning for producing continuous nanofibers. A high-voltage electric field is applied between a needle (connected to a polymeric solution reservoir) and a collector on which polymeric nanofibers are attracted.

Various nanofabrication techniques that are already well established in the semiconductor and micro-electronics industry have been adopted and are constantly growing into the tissue engineering and regenerative medicine field [32]. Many of these techniques rely

on conventional light-/photon- or particle radiation-based lithography mostly in polymeric "(photo)resists" [33,34], or the same/similar methods followed by a transfer of the lithographic pattern through etching or electroplating/galvanoforming. These techniques were not originally developed for biological applications, and the materials commonly used are not dedicated/designed biomaterials or in some cases even cytotoxic, therefore, an increasing body of work is being (and needs to be further) carried out to identify suitable biomaterials and fabrication procedures for biomedical applications. Additionally, nano-lithographic "master" structures are typically too time consuming and costly concerning their (clean room) fabrication to be directly used as disposable culture substrates. Therefore, nanostructures for cell studies are usually created by copying/replicating the pattern(s) of the master into another material [33], directly or via an intermediate template. This replication can be achieved via several techniques, such as soft-lithography [35–37], nanoimprint lithography (NIL) (including hot-embossing/thermal NIL and UV/photo-NIL) [38] and (micro-)injection molding [39–41]. These processes enable not only the replication of structures onto more biocompatible or active materials, but also large-scale production at low cost. Table 1 shows representative studies that combine advanced nanofabrication methods and pattern replication techniques to obtain features <1 µm, a size regime that is of specific interest for cell studies.

Recently, two-photon polymerization (2PP) lithography, the process of crosslinking photosensitive resins with a focused femtosecond laser beam, showed promising results for mimicking biologically relevant architecture with a possible feature size of <100 nm. In contrast to other fabrication techniques where substrate surfaces can be modified to obtain patterns consisting of simple grooves and ridges (also referred as to $2\frac{1}{2}$D nanotexture-type structures), 2PP (laser) lithography allows us to create real 3D nanostructure, which can include, for instance, hollow spaces. The recent deployment of 2PP lithography for an increasing number of biological applications has in turn motivated further improvements of this technique and the development of novel biocompatible materials for new applications [42,43]. The recent advances achieved with this technique allowed the generation of tissue-like structures, such as Descemet's membrane [44], luminal walls of blood vessels [45], limbal stem cell niche [46], cardiac ECM [47] and other 3D biologically relevant models [48–50].

Apart from lithography techniques, electrospinning has had an enormous impact on regenerative medicine by producing structures that more closely mimic nanofibrous ECMs present in in vivo tissues [51,52]. Electrospun fibers have been investigated as promising scaffolds particularly to promote cell growth by mimicking native collagen fibrils in ECM [53,54] as well as mimicking interface tissues [55].

The combination of different patterns and structures can be of particular relevance for hCECs regeneration. For instance, overlaying nanofibrous topographies to hexagonal patterns represents a potential approach to mimic the Descemet's membrane/corneal endothelium interface. More recently, the combination of diverse manufacturing techniques for complex, hierarchical tissue constructs on multiple scales is gaining momentum, and these have been reviewed elsewhere [56]. For corneal applications, applying innovative post-processing techniques, such as microthermoforming [57–59], also opens new opportunities for such hierarchical cell–material constructs, which will help to construct tissue-mimicking grafts with even superior resolution and reliability.

Table 1. Examples of fabrication methods to fabricate sub-micron features used in cell studies.

Fabrication Method	Feature Dimension (nm)	Cell Type	Reference
Electron beam lithography and solvent casting	Width: 20 nm–1 μm Depth: 5–350 nm	Rat dermal fibroblasts	[60]
Electron beam lithography and hot embossing	Diameter: 35–120 nm	Primary human fibroblasts	[61]
	Diameter: 120 nm Interval: 300 nm Height: 100 nm	Human osteoprogenitor and mesenchymal stem cells	[62]
	Diameter: 120 nm Interval: 300 nm Depth: 100 nm	Human mesenchymal stem cells	[63]
	Diameter: 35–120 nm Interval: 100–300 nm	Human fibroblasts and rat epitenon	[64]
Photolithography and soft lithography	Diameter: 1 μm Interval: 600 nm Height: 800 nm	Mouse 3T3 fibroblasts	[65]
Interference lithography and deep reactive ion etching	Pitch: 230 nm Height: 50–600 nm	Human foreskin fibroblasts	[66]
Interference lithography and nanoimprint lithography	Width: 200 nm Interval: 700 nm	Human osteoblasts	[67]
Nanoimprint lithography	Pitch: 420–800 nm Height: 0–350 nm	Murine preosteoblasts	[68]

3. The Impact of Bio-Instructive Nanosubstrates for Culture on Corneal Endothelial Cells

Intuitively, recreating the native Descemet's membrane structure is one of the most effective strategies to generate nanotopographies that can provide essential biophysical cues for the expansion of endothelial cells and maintenance of their phenotype. Since a direct replication of the native tissue, for instance via polymer casting on ex vivo tissue, could be technically complex to accomplish, Gutermuth and coworkers recreated Descemet's membrane-like topographies by a two-step process [44]. Firstly, rabbit Descemet's membrane topography was obtained by peeling and decellularization using ethylenediaminetetraacetic acid (EDTA) and subsequently scanned using confocal microscopy; this process highlighted the hexagonal structure of the Descemet's membrane with irregularly shaped honeycomb features having maximum depressions of 1 μm and lateral size of 20 μm. These topographical data were, thereafter, used to fabricate polydimethylsiloxane (PDMS) substrates with micro- and nanofeatures by two-photon lithography followed by soft-lithography. In this study, the authors demonstrated that human mesenchymal stromal cells (hMSCs), isolated from skin, could be differentiated towards an endothelial phenotype when cultured on such Descemet's membrane-like substrates using Dulbecco Modified Eagle Medium with 4.5 g/L glucose, glutamine without pyruvate (DMEM) supplemented with fetal bovine serum. These cells were able to grow in a confluent monolayer, exhibiting the characteristic polygonal morphology of hCECs, in contrast to the fibroblastic morphology typical of MSCs. The cells cultured on Descemet's membrane replicas also expressed key markers of hCECs such as ZO-1, Na$^+$/K$^+$-ATPase, both at protein and mRNA level. The additional use of collagen coating on PDMS Descemet's membrane-like structures (collagen also supports hCECs in the native Descemet's membrane) induced further expression of hCEC-specific markers such as *COL-8A2* and *PITX2*, therefore showing enhanced control over the cell fate. The possibility to efficiently switch from mesenchymal to endothelial-like cell phenotype using physical factors underlines the correlation of cell phenotype with the physicochemical microenvironment. Such phenotypical transition caused by nanotopographies can be considered remarkable if compared to the opposing undesired endothelial-to-mesenchymal transition generally occurring when attempting to expand primary hCECs on conventional 2D cell culture substrates. In clinical applications, the reversal from a pathological to a physiological hCECs phenotype is also observed in procedures such as Descemet's membrane transfer (DMT) [69], where a replacement of the Descemet's membrane is sufficient to induce the regeneration of host hCECs in vivo,

and partially in descemetorhexis, without endothelial keratoplasty (DWEK), where the complete removal of a compromised ECM environment can also induce self-regeneration of a functional endothelium even without implantation of a new graft [70].

Similar to the hexagonal lattice of the Descemet's membrane, collagen nanofibers constituting the native ECM (Figure 4) are also considered for delivering essential biophysical cues that control corneal endothelial cell phenotype.
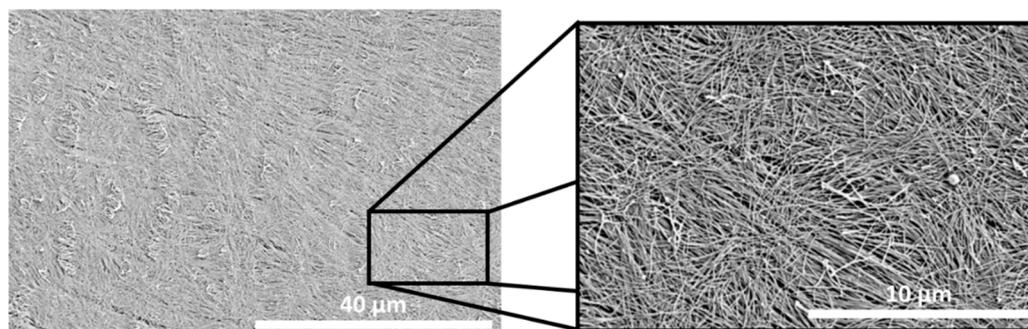

Figure 4. Scanning electron microscope (SEM) images of the human Descemet's membrane obtained by decellularization of the corneal endothelium.

To mimic the nanofibrillary contact guidance of native Descemet's membranes, instructive cell substrates can be produced by electrospinning using different materials. The Fuchsluger group proposed synthetic blends composed of poly(glycerol sebacate) and poly(e-caprolactone) (PGS/PCL) as nanofibrous scaffolds for the culture of corneal endothelial cells [71,72]. The scaffolds had elastic moduli in the same order of magnitude of native corneas. In these studies, a weight ratio of 4:1 parts of PGS with respect to PCL showed the best performances in terms of reaching confluence and exhibiting typical hCEC morphology. The scaffolds were made of aligned fibers with diameters between 200 and 500 nm. Such dimensions were reported to improve cell response when compared to fibers with thickness in the micrometer range [73], confirming the efficacy of nanotopographies for hCECs. While the aligned fibers of these scaffolds induced immortalized hCECs (HCEC-12) to align along their main axis, the authors reported the eventual formation of a confluent cell monolayer with typical hexagonal pattern. Cyto- and immune-compatibility was assessed by studying the interaction of the scaffolds with peripheral blood mononuclear cells and polymorphonuclear leukocytes. Similarly, Kruse and coworkers also investigated the cytocompatibility of electrospun scaffolds made of poly(methyl-methacrylate) (PMMA), poly(lactic-co-glycolic acid) (PLGA) and PCL [74]. Generally, PMMA electrospun meshes exhibited high cytotoxicity, while both PCL and PLGA showed good levels of biocompatibility. Regarding the topographical features, although keeping equal fabrication parameters for the different materials, differences in fiber diameter and interstitial spaces were noticed. Cells preserved their normal morphology only on smaller fibers with smaller interstitial space, which was obtained with PLGA although perfect cellular monolayers could not be generated with any of the materials tested.

In another study, monolayers of the human corneal endothelial cell line B4G12 could be successfully formed using blends of nanofibrous structures made of silk fibroin and poly(L-lactic acid-*co*-ε-caprolactone) (P(LLA-CL)) [75]. The authors stated that the combination of natural and synthetic materials offered by silk:(P(LLA-CL)) constructs could provide both biocompatibility and mechanical properties necessary for the culture of endothelial cells. This study evaluated different parameters such as cell adherence, proliferation and gene expression as well as light transmittance, identifying 25:75 as the optimal ratio of SF:P(LLA-CL) for the culture of corneal endothelial cells.

Yim's group extensively studied bio-instructive substrates replicating features such as pillars and wells of various dimensions in different materials (Figure 5) [76–81]. In particular, they were able to improve not only hCECs culture but also to recreate a disease

model such as Fuchs' endothelial dystrophy by using these topographies (Figure 5B), which is the main indication for endothelial keratoplasty (surgery) worldwide and for which currently no functional in vitro models are available [80]. Interestingly, the use of substrates with higher pillar density and with larger features (>10 μm) was shown to prevent monolayer formation and induce Fuchs' endothelial dystrophy conditions (i.e., mimicking guttata), stressing the importance of sub-cellular features for physiological hCEC expansion and phenotype.
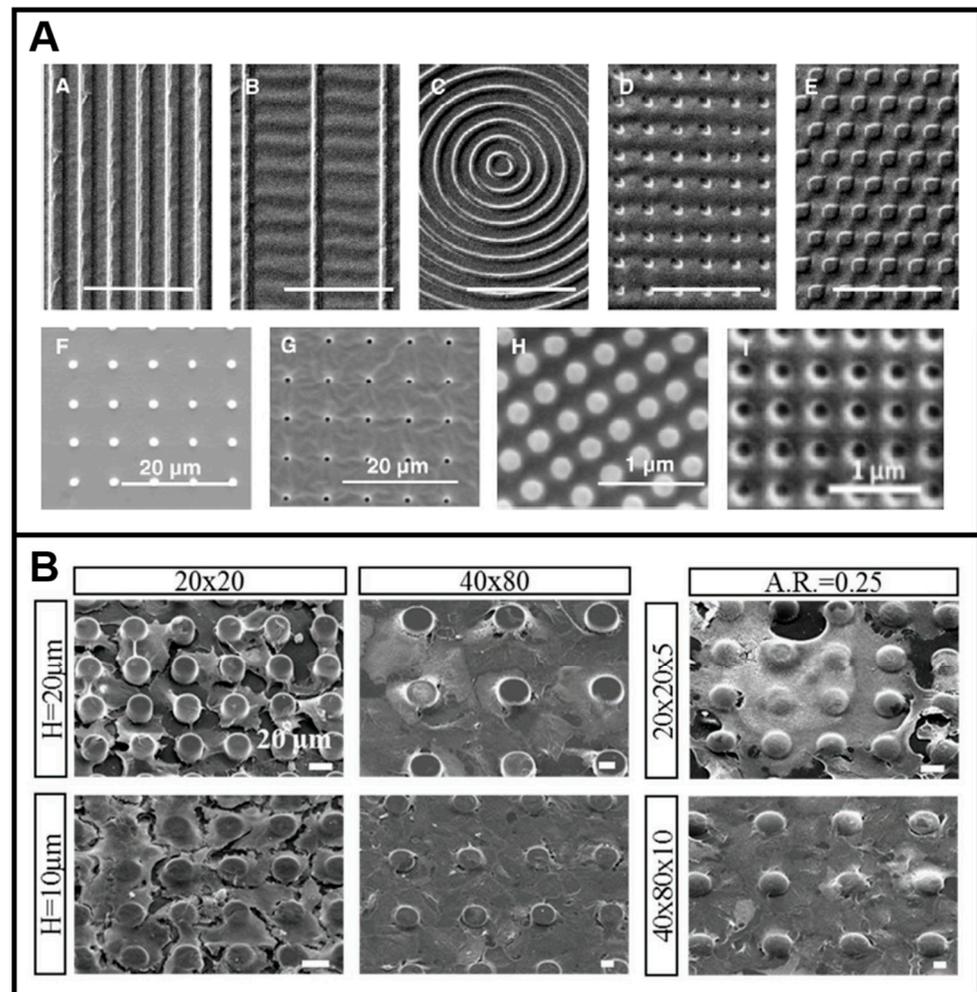

Figure 5. Examples of substrate topographies used for the culture of hCECs. Nanopillars (**E,F,H** in top image) and nanowells (**D,G,E,I** in top image) were demonstrated to better induce hCECs expansion and maintenance of their phenotype compared to other topographies (**A–C** in top image) (**A**), while patterns with larger dimensions can be used for modelling disease of hCECs such as Fuch's endothelial dystrophy (**B**). Scale bars: 20 μm unless specified. Reproduced from [77] (**A**) and [80] (**B**).

Regarding the influence of particular patterns on hCECs expansion and phenotype, nanopillars and nanowells showed improved guidance compared to other nanopatterns [76,77]. Pillars patterned on gel-methacrylate (GelMA) substrates induced enhanced ZO-1 expression and morphological parameters according to whether they were arranged in a square or hexagonal pattern [81]. The authors also showed that the expression of ZO-1 could be retained upon removal of the biophysical cues, i.e., when cells were subsequently cultured again on conventional tissue culture plastic, indicating a memory effect of cells after exposure to topographical cues [79].

Nanotopographies can also be used to generate carriers for tissue engineered corneal endothelium, therefore not only for ensuring hCECs expansion in vitro but also for main-

taining the hCECs phenotype upon transplantation. In a study by Kim and coworkers, nanoporous (pore size <200 nm diameter) silk fibroin films were explored as a carrier for rabbit corneal endothelial cells [82]. Although this study was not focused on comparing different nanofeatures but assessed the effect of sericin content in the films, it demonstrated that this nanomaterial supports cell attachment and morphology well, as well as expression of ZO-1 and Na^+/K^+-ATPase both at the mRNA and protein level.

4. Conclusions and Future Directions

Effective cell therapy and regenerative medicine approaches require hCECs to form confluent monolayers, while maintaining their original phenotype. To induce the formation of a functional, confluent hCECs monolayer, there is a limitation on the features and dimensions of topographies that can be used. In particular, topographical features (both lateral size and height), with dimensions comparable to or larger than the cellular size (i.e., $\geq$2–5 μm), can hinder the formation of a continuous layer of cells and steer cells away from the corneal endothelium phenotype. For this reason, subcellular (nano-)topographical patterns have demonstrated better support for the physiological hCECs' phenotype over micro-topographical patterns. In light of the studies reviewed in this work, it is evident that nanotopographies are a potential technology to further support primary hCEC expansion and, therefore, increase the pool of cells suitable for corneal endothelial regenerative therapy, while larger micro-topographies could provide avenues for modelling hCEC disease.

While nanofabrication techniques enabled the generation of improved substrates for hCECs culture, further improvements are required. For instance, imprinting nanoto-pographies on electrospun fiber meshes by fiber-level patterning results in hierarchically structured substrates, which could improve current protocols for hCECs regeneration [83]. Moreover, the combination of multiple nanotechnologies that allows the superimposition of porosity onto nanopatterns can also generate new strategies for recapitulating the native endothelial environment and facilitate nutrient, water, ion and metabolite transport of tissue engineered corneal endothelium. Creating controlled porosity on substrates is possible by various methods, such as photolithography and ion-track technology [84], the latter being already implemented to fabricate porous polycarbonate membranes, which are commercialized for cell culture applications [85]. On the other hand, self-assembly techniques such as water droplet-assisted methods [86] including breath figure [87] and phase separation methods [88] can still be considered as alternative to lithography techniques.

The combination of biomaterials, fabrication techniques and specific patterns could impact on the overall biophysical and biological performances [89–91]. Biomechanical properties, biodegradability as well as ions and nutrient diffusion, for instance, are all important factors needing careful consideration when selecting suitable materials and fabrication techniques for translational applications. Moreover, major steps for the design of future studies also include the optimization of surface chemistry and substrate stiffness.

Thus far, nanotopographies demonstrated efficacy towards inducing corneal endothelial regeneration; however, new (combinations) of existing nanotechnology offer the potential to provide additional tools for further advancing this field. The studies reported in this review can, therefore, provide basic insights to design novel studies for hCECs regeneration and eventually provide therapeutic solutions for corneal endothelium dysfunctions.

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