Review Article

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Reconstruction and Regeneration of Corneal Endothelium: A Review on Current Methods and Future Aspects

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Abstract

Corneal transplantation is a popular choice of treatment for many corneal disorders. However, the shortage in availability of donor tissues limits the amount of corneal transplantations around the globe. Continuous increase in the number of patients has boosted the need for clinical grade tissues or a reasonable substitute to overcome this issue. Alternatives include cell, tissue or bio-engineering, cell culture *in vitro*, the potential use of stem cells or other related therapies. There is a very rapid advancement in the field of endothelial keratoplasty as it has advantages being a less-sutured surgery with quicker rehabilitation and better post operative visual outcomes. Thus, to overcome the necessity of donor tissues for selective keratoplasty, the endothelial reconstruction or regeneration is currently studied. This review highlights recent advances in the isolation, culture, expansion of the corneal endothelium and the use of scaffolds or matrices to facilitate endothelial transplantation.

Keywords: Human corneal endothelium; Keratoplasty; Neovascularisation

Introduction

Human cornea is a highly organized transparent tissue and is well determined by its functional layers as shown in (Figure 1). The posterior layer of the cornea is the endothelium which is a physiologically important monolayer of cells. Human Corneal Endothelium (CE) plays a major role in maintaining the corneal transparency, thickness and hydration [1,2]. As CE dysfunction is the second leading cause of corneal blindness and the human Corneal Endothelial Cells (CECs) are non-regenerative, its preservation and maintenance becomes a critical issue. The CECs have a low tendency to proliferate *in vivo*, as they are arrested in the G_1 phase, and therefore these cells spread out to replace the deceased cells, thus maintaining the functional integrity and corneal deturgescence [3,4]. Accidental or surgical trauma can result into acute corneal endothelial dysfunction which results in the inability of the pumping function of the endothelium which is necessary for the drainage of excess fluids. This causes critical anomalies like stromal edema, loss of transparency and most importantly the visual acuity which usually leads to the clinical condition of bullous keratopathy [2]. The corneal endothelium is assumed to be originated from the neural crest cells. It has already been described that the neural crest cells migrate and differentiate during the developmental phases of the cornea. Periocular mesenchymal cells are originated from the neural crest cells and are responsible for the development of the corneal epithelium and the synthesis of the primary stroma. These cells further migrate to the periphery of the optic cup eventually moving between the lens and the corneal epithelium for the development of the trabecular meshwork and the corneal endothelium. Further, in the second phase of the development, the neural crest cells occupy the primary stroma and differentiate into corneal keratocytes [5]. CECs are metabolically active and continuously functions as a fluid pump for the movement of fluid from the stroma and the anterior chamber. Thus, as described earlier, the endothelial layer plays a significant role, but lacks the capacity to regenerate and hence its viability should be maintained. Currently, surgical replacement of the diseased endothelium by a healthy donor's tissue using corneal transplantation is the only solution to restore vision [6]. However, the availability and quality of corneas are main concerns; more than 40% of the donated corneas to the Veneto

Eye Bank Foundation (FBOV – Venice, Italy) are rendered unsuitable for transplantation. Additionally, more than 60% of the donors are over 60 years old which may lead to graft rejection in the preliminary phases of evaluation due to low endothelial cell density.

Isolation, culturing of the cells and tissue engineering, thus, can play a vital role in the treatment of endothelial dysfunction. It has also been shown that the cultured cells are easier to produce using sphere-forming assay as compared to adult stem cells. It was also observed that the CE periphery possess a higher density of precursors which have a strong proliferation capability, as compared to the central endothelium and can be differentiated into neural or mesenchymal cells. Using sphere-forming assay, a mass production of CECs precursors can be achieved, which could ultimately reduce the waiting lists of the patients requiring endothelial keratoplasty [5].

The current treatment available for the restoration of the corneal endothelium is keratoplasty, which represent more than 22% of the corneal tissues supplied by FBOV in 2012. This procedure is hindered by several difficulties, including the shortage of donor tissues, post-surgical complications associated with the use of drugs to prevent immune rejection, and a significant increase in the occurrence of glaucoma. Recently, surgical procedures such as Descemet's Stripping Automated Endothelial Keratoplasty (DSAEK) have focused on the transplantation of corneal endothelium, yielding better clinical results, yet the shortage in donor tissue remains a main issue [7]. The emerging strategies in the field of cell biology and tissue cultivation of corneal endothelial cells aim
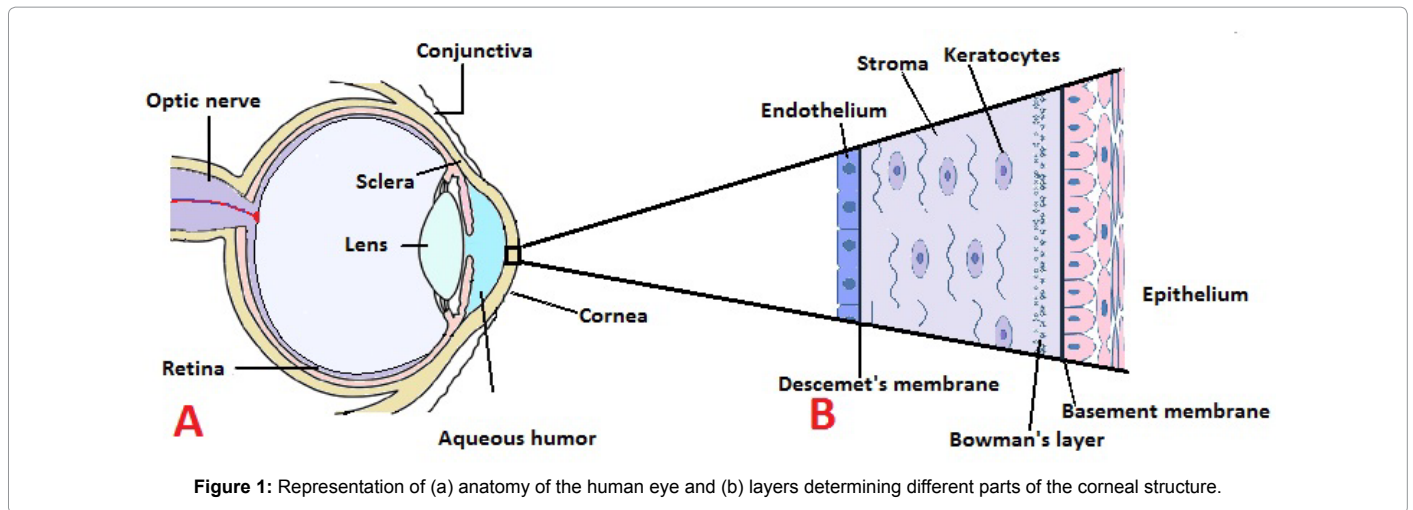
Figure 1: Representation of (a) anatomy of the human eye and (b) layers determining different parts of the corneal structure.

at the production of transplantable endothelial cell sheets. Cell therapy focuses on the culture of corneal endothelial cells retrieved from the donor followed by transplantation into the recipient. This strategy likely poses multiple advantages over the conventional penetrating keratoplasty, such as post-operative complications like irregular astigmatism, wound leakage, corneal infection, neovascularisation and epithelial defects. Recently, the focus is on overcoming the challenge of harvesting human corneal endothelial cells and the generation of new bio-membranes to be used as cell scaffolds in surgical procedures. Current results represent important progress in the development of new strategies based on alternative sources of tissue for the treatment of corneal endotheliopathies. Similarly, multiple techniques have been described in the literature [8], which will be the focus of this review.

Isolation of the Human Corneal Endothelium

Corneal endothelial isolation method has been followed ubiquitously, even if the majority of the researchers rely on manual dissection. Engelmann et al. described a method in which whole corneas were digested with collagenase or trypsin. This method inevitably caused contamination of corneal fibroblasts in CECs culture, a problem that Engelmann solved by culturing cells in selective L-valine-free medium culture [9].

Initial techniques for isolation of CECs include stripping of the Descemet's Membrane (DM) and the endothelial explants from the cornea and direct culturing in T-25 flasks in complete medium, as described in the initial protocols for primary CEC cultures. It was observed that the cells were easily cultivated from the explants and after 10-20% surface confluency, they were trypsinized and re-cultured [10,11]. Various studies have shown other techniques of isolation of CECs using the 'peeling off' method, as described by Engler et al. [9]. As per this procedure, the DM with endothelial cells are peeled off and incubated in growth medium. Treatment with 0.02% Ethylenediaminetetraacetic Acid (EDTA) or trypsin-EDTA is carried out to loosen cell-cell junctions. The loosened cells are finally detached from the DM by passing through a flamed-polished pipette.

However, most recent protocols have shown scrapping of CECs with the DM using a surgical blade [11]. Although this method may eliminate the fibroblast contamination, scrapping of cells using a blade will reduce the viability of the primary cells dramatically [12,13]. As per our experience, the big bubble technique using air as a medium of separation can be used for isolation of the Descemet's membrane and

endothelium to reduce the damage caused during manual methods like scraping. The big bubble method is a simplified technique where the air is injected using a syringe between the DM and the stroma to separate the two layers and only obtain the DM and endothelium. In our hands, we found that scrapping cells is only useful when performing experiments not requiring the maintenance of the integrity of the endothelial cells, such as DNA extraction. Currently, many CEC isolation protocols involve two steps, peel and digest. In the first step, central cornea is separated by trephining 8-mm diameter. Further, CECs are stripped from the posterior surface of the peripheral corneo-scleral tissue under a dissecting microscope. Stripping of CECs can also be performed with the aid of a vacuum suction holder, as described by Peh et al. [14]. Vacuum suction created by a pump, in fact, helps to stabilize and hold corneal tissue in place for further cutting [12]. After peeling, the Descemet's membrane and endothelial layer are finally digested enzymatically, using dispase or EDTA, followed by pipetting [13]. However, enzymatic digestion requires a prolonged incubation time to detach cells from the matrix, subsequently leading to higher cellular degeneration [12]. Therefore, enzymatic reaction method could be followed as per the studies described by Li et al. [13] and Peh et al. [14] who proposed 2 mg/mL collagenase A digestion which result into high viability and expression of tight junction protein Zonula Occludens-1 (ZO-1) and gap junction protein connexin-43 [12]. Collagenase digestion allows the recovery of CECs that can be dissociated into single cells by a lower incubation time of 5 minutes using trypsin digestion [10].

Thus, it is evident that there is no standardized method to isolate the CECs from the donor corneas. However, researchers developed personalized manual systems to excise and culture the CECs. Further research is on-going to find the most appropriate way to isolate complete tissue maintaining the high viability of the cells for further use.

CE Cell Culture *In Vitro*

Various artificial matrices have been reported to promote cell attachment and growth of CECs. Engelmann et al. suggested the use of extracellular matrix produced by bovine corneal endothelial cells [15]. Also, chondroitin-sulfate and laminin [12], laminin-5 [16], extracellular matrix secreted by bovine corneal endothelial cells [17,18], and fibronectin plus type I collagen coating mix [19] have been used for culturing CECs. Particularly, studies with recombinant laminin 5 and its receptor α3β1 suggested that this factor may play a critical role

in promoting CEC culture and may contribute to the practical use of tissue engineering [16,20]. It has also been reported that proliferation of CECs can be influenced by the presence of various growth factors like Fibroblast Growth Factor (FGF) [11,21], Epidermal Growth Factor (EGF), Nerve Growth Factor (NGF) [20], and Endothelial Cell Growth (ECGF) supplements [10,17].

Different culture media have been used for CECs like Dulbecco's modified Eagle's medium (DMEM), Opti-MEM-I, DMEM/F12, and Ham's F12/M199. Engelmann et al. tested 25 different nutrient media and found that F199 (a 1:1 mixture of M199 and Ham's F12) was the most efficient basal medium in promoting the growth of CECs. Further the group studied six different serum types and found that human serum and fetal bovine serum showed optimal growth promoting activities in combination with F99 media. They also reported that at low serum concentration, FGF, or alternatively EGF, was necessary for promoting the growth of CECs. Peh and colleagues evaluated the effect of DMEM, Opti-MEM-I, DMEM/F12, and Ham's F12/M199 culture media in propagation of CECs and they confirmed that CECs cultured in Ham's F12/M199 are significantly more proliferative and expressed markers characteristic of human corneal endothelium (Na+K+/ATPase and ZO-1) [14].

Recently, a culture method using basic FGF (bFGF) and L-ascorbic acid-phosphate (Asc2-P) has been developed. This method seems to protect cells from apoptosis, perhaps by diminishing intracellular Radical Oxygen Species (ROS) generation. This culture method helped cells to maintain their characteristic polygonal morphology longer than other traditional cell culture methods [21]. Mergler et al. studied the expression and function of Transient Receptor Potential (TRP) channels of the Vanilloid (V) isoform subfamily in CECs. It was observed that the *temperature-dependent activation* of TRPV3 channels may play an important role in CECs function [22,23]. Enomoto et al. established a two steps culture method where the CECs were first maintained in Opti-MEM or low glucose DMEM containing 8% fetal bovine serum and bFGF, until they reached confluence. The cells were then cultured in medium without supplements in order to stabilize the monolayer and optimally reflect its *in vivo* morphology [24].

Serum-free conditions for CECs have also been developed to avoid any possible contamination with microorganism and prions, and eventually more suitability for clinical applications of CECs [12,24]. Okumura et al. [25] demonstrated that the use of Y-27632, a compound that selectively inhibits Rho and Rho-associated Kinases (ROCKs), promotes the adhesion of cynomolgus monkey CECs (MCECs) and inhibits apoptosis. Although MCECs show cell-senescence phenotype after few passages, the MCECs were successfully cultivated in the presence of Y-27632. Therefore, it was determined that ROCK inhibitor may serve as a new tool for cultivating CECs [25].

Schmedt et al. [26] demonstrated the existence of morphologically distinct subpopulations in cultured CECs that grow in colony-like structures and are smaller than the rest of cells. These cells have high proliferative potential, dependent on endogenous upregulation of telomerase. By performing a selective isolation of those morphologically distinct cells, Schmedt et al. established long-term corneal endothelial cell cultures. They also transfected the isolated cells with human Telomerase Reverse Transcriptase (hTERT), which further increased its proliferative capacity. Transduced cells retained corneal endothelial cell characteristics and functionality. They maintained baseline synthesis of p53 and readily upregulated phospho-p53 during oxidative stress and did not over-express oncogenes. These cells may be a useful source for propagation of CECs *in vitro* and for the study of endothelial cell biology

and development of cell-based therapies [26]. Alternatively, CECs can also be triggered to proliferate *in vitro* as described by several groups however, this basic research is still distant from practical application [27].

Since CECs are derived from Neural Crest Cells (NCC), Ju et al. [28] proposed that these cells can serve as a possible source of cultured CECs for bioengineering applications. NCC can be isolated not only from fetuses, but also from adult skin and hair follicles [8]. Ju et al. [28] further described an experimental protocol that effectively differentiated rat embryonic NCC to corneal endothelial cells. Briefly, NCC was isolated from embryos at embryonic day 9. The neural tube was dissected at midbrain level and directly cultured cranial segments in a petri dish coated with fibronectin. Cells that migrated out of the explants were harvested and cultured for seven days in a differentiation medium, made up of a 3:1 mixture of DMEM and F12, with 10% FBS, in petri coated with 10 μg/ml laminin and 10 mg/ml chondroitin-6 sulphate. Two weeks after induction, cells showed characteristic endothelial polygonal morphology and expressed cadherin and transcription factor Fox C-1 and Pitx-2 that are closely related to corneal endothelial cells' development [28]. Basic methods of media preparations are described in Table 1 [10,12-14,16 ,17,21,29-38].

Three Dimensional Systems

Although CEC culture is useful in the early stages of investigations, it has been observed that it cannot reproduce the complex interactions between the different cell types in the cornea. To mimic the response of cornea *in vivo*, wound healing, the effect of toxins and therapeutic agents can be studied using *in vitro* culture models. Therefore, the first three-dimensional organ culture system for cornea was developed by Brunette et al. In this system human corneas were placed endothelial side down and were perfused with DMEM and high glucose at a rate of 5 μL/min in a perfusion chamber with a constant pressure of 18 mmHg. Forty human cadaver donor corneas with an average thickness of 697 μm (considering the average normal human corneal thickness at 520 μm) were perfused. The first week of perfusion showed thinning of corneas, although the thickness remained higher than the average. After perfusion at 37°C for a week the endothelial cells size (polymorphism was observed) increased, suggesting a cell loss in 6 out of 10 perfused corneas. The effect of mechanical removal of endothelial cells and treatment with ouabain which is an inhibitor of endothelial pump was also evaluated. The corneas where the endothelium was removed, swelled and increased in thickness within two hours, later the thickness remained constant. Instead, corneas exposed to ouabain swelled and gradually lost their endothelial cells, eliminating endothelial barrier [39].

Foreman et al. [40] developed a technique where the anterior chamber was maintained by agar-collagen gel. In this system endothelial concavity was filled with an agar-collagen mixture before corneo-scleral rings were cultured in Medium 199. This system was used to study the effects of growth factors on re-epithelialization of wounded corneas. Therefore, the examination and maintenance of endothelial layer was not studied in this culture system.

Recently, a perfusion chamber has been described which irrigates epithelial cells and perfuses endothelium separately. In this system, a cornea is clamped in a horizontal plane and the epithelial surface of the cornea is exposed to air in a humid chamber. Corneas were perfused at a flow rate of 1 ml/min and the reservoir was elevated above the level of the cornea to create a positive pressure of 18 mmHg that simulates physiological conditions. A brass heat exchange block through which

Base Medium	Serum	Glutamine	bFGF	NGF	EGF	Insulin	Transferrin	Others	Antibiotics	References
MEM	10% HS					5 µg/mL	5 µg/mL	5 ng/mL sodium selenite, 150 g/mL ECGS	100 U/mL penicillin, 100 g/mL streptomycin, 0.25 g/mL amphotericin B	Blake et al. 1997
MEM	5% CS+10% FBS	200 mM						2% esssential aminoacids, 1% non essential aminoacids	10 g/mL gentamicin, 1,2 g/mL amphotericin B	Yue et al. 1989
Low glucose DMEM	15% FBS		2 ng/mL						2.5 mg/L amphotericin B, 2.5 mg/L doxycycline	Yamaguchi et al. 2011
DMEM	10% FBS		2 ng/mL						50 U/mL penicillin, 50 g/mL streptomycin	Ishino et al. 2004
DMEM	15% serum	30 mg/L L-glu	2 ng/mL						2.5 mg/L doxycycline, 2.5 mg/L fungizone, 30 mg/L L-glutamine	Miyata et al 2001, Amano et al. 2003
DMEM	15% FCS		2 ng/mL					Asc-2P 0.3 mM	NM	Shima et al. 2011
DMEM/F12	15% FBS				10 ng/mL	5 µg/mL		100 ng/mL bovine pituitary extract	50 mg/mL gentamicin and 1% antibiotic antimycotic solution	Jiucheng et al. 2011
DMEM/F12	20% BCS					1% ITS	1% ITS	1% ITS	1% antibiotic-antimycotic	Cheng 2007
OPTI-MEM-I	8% FBS			20 ng/mL	5 ng/mL EGF			20 µg/mL ascorbic acid, 200 mg/L calcium chloride, 100 g/mL pituitary extract, 0.08% chondroitin sulfate	1X antibiotic/antimycotic, 5 g/mL gentamicin	Zhu et al. 2004, Joyce et al. 2009, Gary et al. 2011, Mimura et al. 2012
EGM-2	10% FBS		Present in BM		Present in BM			IGF, VEGF present in BM	Gentamicin, amphotericin B	Choi et al. 2010
M199	20% HuS	4 mM						400 µg/mL ECGS	100 g/mL penicillin, 100 g/mL streptomycin	Pitsov et al. 1988
F99:Ham's F12 and M199 (1:1)	5%FBS		10 ng/mL			20 µg/ml	2.5 µg/mL	20 µg/mL ascorbic acid, 0.6 ng/ml sodium selenite	NM	Engelmann et al. 1995, Engelmann et al. 1989, Gary et al. 2011

EGM: Endothelial Growth Medium; BM: Base Medium; NM: Not Mentioned; MEM: Minimum Essential Medium; DMEM: Dulbecco's Modified Eagle Medium; HS: Horse Serum; FBS: Fetal Bovine Serum; CS: Calf Serum; FCS: Fetal Calf Serum; BCS: Bovine Calf Serum; HuS: Human serum; bFGF: basic Fibroblast Growth Factor; NGF: Nerve Growth Factor; EGF: Epidermal Growth Factor; IGF: Insulin: Like Growth Factor; VEGF: Vascular Endothelial Growth Factor; ECGS: Endothelial Cell Growth Supplement; ITS: Insulintransferrin-Selenium; Asc-2P: L-Ascorbic Acid-Phosphate.

Table 1: List of different constituents for media preparation used by various researchers for culturing corneal endothelial cells in vitro [10,13,14,16-18,22,30-39].

warm water can be circulated ensured that chamber temperature was 35°C. Thiel et al. [41] proposed this chamber as a new tool for toxicity studies. However, as the epithelial irrigation was performed manually, this system was evaluated only for 14 hours [41]. Nonetheless, this system was further modified by Zhao et al. [42] where an automated irrigation system was built in addition. This culture system was able to maintain the epithelium, endothelium, putative limbal stem cells and stroma of Bovine corneo-scleral buttons in good condition over a period of 10 days [42]. Thus, these systems could potentially be used for research purposes to mimic the surgical environment in vitro.

Animal Models for the Study of Re-endothelialization after Keratoplasty

Animal models have been studied widely for in vivo studies before entering human clinical trials. Although various animal models have been used for corneal studies, specificity of the models can sometimes render varied results. Therefore, selecting and obtaining reasonable and reproducible data from animal models is one of the concerns. Rat keratoplasty model is widely used for studying corneal transplantation, immunological responses and re-endothelialization [43]. Particularly, it was found that allogenic transplantation using adult rats of Fisher strain as donors and adult Lewis rats as recipients that always lead to rejection is a reliable model for studying immunological responses [44].

It has been demonstrated that after corneal rejection, allografts could restore transparency and recover the whole endothelial layer. For re-endothelialization after keratoplasty, two possibilities could arise: donor endothelial cells may survive and let recovery of endothelial function, or host endothelial cells may regenerate the endothelial layer. Plskova et al. [45] performed allogenic transplantation of murine corneas and noted that there was an immune response against donor endothelium that caused a loss of endothelial cells during the first 48 hours. After this cell loss, a recovery of endothelial cells on the graft was observed. This suggested that donor cells survived the immune response, enlarged and spread, eventually leading to the recovery of endothelial monolayer. The degrees of endothelial coverage in the grafts showed high variability and in three mice allografts were completely rejected after 14 days and therefore no endothelial recovery was seen.

Hori et al. [46] used a mouse model with Green Fluorescent Protein (GFP) expression in the CECs, and did not observe re-endothelialization after cell loss in rejected allografts. In both syngeneic and allogeneic non rejected grafts, instead, donor endothelial cells were seen in the graft, even if density of endothelial layer gradually declined during the first eight weeks [46]. Gong et al. [47] performed keratoplasty between Dark Agouti rats as donors and Lewis rats as recipients. It was observed that initially all the allografts were rejected and therefore the absence of cells was recorded followed by a continuous increase in the number of cells. Three weeks after rejection, the whole endothelium was covered by CECs and corneal transparency was recovered. However the distribution and cell nuclear shape were not regular after six months whereas EC density was only half of the normal levels.

Schwartzkopff et al. [48] used a double animal model; a rejection immunological group (transplantations using Fisher rats as donors and Lewis rats as recipients) and a surgical group (completely mechanical endothelial abrasion before transplantation). All allogenic grafts were rejected and after a median time of 15 days following surgery, CECs were lost. In less than one month, both groups showed a new endothelial layer with irregular and enlarged CECs. Immunostaining for the proliferation marker Ki-67 detected dividing cells in the area adjacent to the graft and on the graft periphery. Schwartzkopff et al. [48] suggested that after the wound stimulus, CECs can divide, repopulating the scaffold. It was found that only after reduction of leukocytes infiltration, endothelial layer and corneal clarity could be restored and thus the role of immune response in the re-endothelialization is emphasized. It is also assumed that only host corneal endothelium which does not provoke further immune stimulation could restore corneal clarity. However, the regeneration of cells needs to be reconfirmed in terms of the source (donor or recipient).

Precursor Cell Culture and Clinical Applications

Precursor cells are unipotent regenerative stem cells with restricted ability for proliferation. Human and rabbit CEC-derived precursors were first isolated by Yokoo et al. [49] using a sphere-forming assay. The DM with CECs was peeled off from human corneas and cut into small pieces. Overnight incubation in DMEM-F12 with collagenase and then in EDTA solution was performed. Finally they were dissociated into single cells by pipetting. Cells were plated at low density (30-50 cells/μl) in uncoated wells with 40 ng/mL bFGF and 20 ng/mL EGF and with this the cells started forming primary spherical colonies. Colonies expressed β3-tubulin and nestin, indicating that they contained immature and undifferentiated cells. To distinguish growing spheres from dying cell clusters, only those spheres with a diameter of >50 μm were counted [49].

Mimura et al. cultured spheres from New Zealand white rabbit corneas in a basal medium containing a methylcellulose gel matrix to prevent cell re-aggregation. It was observed that when the primary colonies were treated with 0.5% EDTA and incubated as floating culture (small floating spheres), the colonies generated some secondary spheres that had a viability of 90% which also expressed β3-tubulin and nestin. However, third-passage spheres cannot be generated, suggesting that sphere-precursors have a limited self-renewal potential [50]. To promote differentiation, spheres could be trypsinized and cultured in wells coated with poly-L-lysine and fibronectin, using DMEM-F12 with EGF, bFGF and Bovine Serum Albumin (BSA). Cultured cells derived from the sphere, when confluent, have a CEC-like hexagonal shape and transport activity determined using a special chamber for measuring ion transport; the Ussing chamber [51]. The rate of primary and secondary sphere formation was significantly higher in peripheral rather than central cornea, suggesting a higher self-renewal capacity of cells from the peripheral region [51]. As the number of CE precursors that can be isolated from a cornea is insufficient for clinical applications, Mimura et al. also isolated spheres from fourth or fifth passage of cultured CEs. The cultured CECs were incubated with 0.2% EDTA, later dissociated into single cells by pipetting and finally cultured the cells at a density of 10 cells/μl in basal medium. It was found that cultured CECs-derived precursors have a propensity to differentiate into corneal endothelial-like cells and sphere forming assay can enrich young precursor cells from CECs and can be used for mass production for clinical applications.

Using cultured precursors' derived ECs, a novel technique was proposed for the treatment of bullous keratopathy, a pathological condition characterized by corneal endothelial dysfunction and loss. In a rabbit model, the CEC-derived precursor cells were injected in the eye-down position and it was observed that injection of spheres restored the endothelial function and decreased corneal edema [52].

Yamagami et al. [53] seeded progenitors derived from cultured human CEC onto denuded human amniotic membranes. Cultured CECs were seeded at the same density in controls and tests. It was found that precursor cells had a more regular hexagonal shape than the adult CEs, as shown by immunostaining of the tight junctions using ZO-1 [53-55]. Mimura T et al. [54,55] showed a slightly different method of isolation and culture of precursor cells after DM dissection with the intact CE layer. The membrane strips after centrifugation was incubated at 37°C in 0.02% EDTA for an hour. Afterwards, the cells were cultured on an undiluted fibronectin/type I collagen coated plates and incubated in 5% CO_2. This was then sub-cultured in a 1:4 ratio and only the cells from 4th and 6th passages were used. Glass coverslips coated with 50 μg/ml of PLL (poly-L-Lysine) and 10 μg/ml of fibronectin in separate wells were used to transfer the primary spheres. Differentiation was promoted using different concentrations of FBS (1% and 15%) and the cultures were further allowed to grow for another week but it was observed that the cells migrated out of the spheres. Alpha Smooth Muscle Actin (α-SMA), which is a mesenchymal cell marker, showed less than 5% positivity when the cells were cultured with 1% or 15% FBS. However, the cells were found negative for control IgG and for the differentiated epithelial cell markers like cytokeratin 3, nestin, β-III tubulin, and Glial Fibrillary Acidic Protein (GFAP) which is a mature glial cell marker. This shows that a single colony could potentially form a small population of mesenchymal cells under clonogenic conditions. Using Real Time Polymerase Chain Reaction (RT-PCR), the expression of nestin and α-SMA positive cells was confirmed. Where β-III tubulin mRNA positive cells were detected in cultures with 1% FBS, the immature cell marker, neuronal marker and mature glial cell marker nestin, β-III tubulin and GFAP respectively were expressed only in the spheres from donor CEC while their progeny expressed β-III tubulin and nestin, but not GFAP. However, no expression of neuronal markers and decreased expression of immature cell markers were observed in the spheres and progeny obtained from these cultured CECs. This indicated that the differentiation of the precursors occurred during culture. Therefore, considering the tissue or cell regeneration or transplantation, it was determined that use of the precursors obtained from cultured CECs can be a much better option rather than using the donor CECs directly [54,55].

Huang et al. [56] described a new hanging-drop method to generate sphere colonies from cultured CECs. In this method, cells were suspended in droplets of medium, where they develop into coherent 3D aggregates. In a bovine model, Huang et al. [56] found that frequency of sphere formation using hanging drop was higher than using sphere-forming assay. Not all cells that reside in the spheres displayed the same ability to proliferate at a clonal level, but some cells did not divide and some divided to give rise to various sized colonies of endothelium. So a hierarchy of BCFCs was determined with various levels of proliferative potential and found that it was quite similar to that observed in bovine vascular endothelial cells.

Bioengineering, Reconstruction and Transplantation of Endothelial Sheet

The earlier attempts for reconstruction of a full-thickness cornea were performed onto the DM of bovine, rabbit and human corneas

denuded of CECs. The CECs were cultured on DMEM and then seeded onto these denuded DM scaffolds or stromal discs [57]. Also human amniotic membranes have been used as a carrier for cultured CECs [29], but because it is not fully transparent, it is not highly suitable for clinical applications. Gospodarowicz et al. [58-60] seeded bovine CECs onto rabbit corneas denuded of their endothelium. It was observed that when corneas were transplanted back into rabbits, the corneal buttons remained clear and showed no edema, which suggested that in this culture system, bovine CEs maintained their function [58-60]. More recently, several methods for culturing CECs on synthetic or biological grafts have been established. Choi et al. [29] seeded CECs onto decellularized thin-layer of corneal stroma [48], whereas Honda et al. seeded cultured CEC suspensions onto human corneal stromal discs and transplanted the grafts in rabbit model. Corneal edema was observed after transplantation, but the corneal transparency was recovered and the edema decreased in less than one month [57]. Ishino et al. [30] transplanted the grafts in rabbits and shown only little edema and corneal clarity.

Mimura and colleagues reconstituted CEC sheet seeding CECs onto a graft composed of a network of loosely cross-linked type I collagen fibers. They developed a method to examine the functionality of their graft. Because transparency of human cornea depend primarily on sodium and bicarbonate ion transport driven by Na+/K+pump, they examined the pump function in reconstructed CE sheets by electrophysiological measurements. Using a Ussing chamber EC pump activity was measured which mainly depends on Na+, K+-ATPase to confirm Na+, K+-ATPase activity [31,61].

However, Mimura et al. [61] labeled cultured CECs with the fluorescent dye 'DiI' and seeded cells on collagene sheets. EC sheets were coated with a silicon plate. Descemetorhexis was performed on rabbit eyes and the grafts were transplanted similar to current DSAEK procedures. DM was peeled in the control group of rabbits. Corneal edema was decreased rapidly after transplantation in DSAEK groups. After 28 days, DSAEK corneas recovered clarity and were thinner than corneas of control groups. Fluorescence microscopy showed numerous DiI-labelled cells on the posterior corneal surface, while frozen sections showed a monolayer of DiI-labelled cells on Descemet's membrane [61].

Koizumi and colleagues transplanted monkey EC sheets cultured on collagen type I carriers [62] into monkeys' eyes, but they found that the corneas recovered their clarity only six months after transplantation.

Other investigations showed that biological tissue equivalent products could be created if the well-organized bioengineered human corneal endothelial cell monolayers are collected from thermo responsive cultures. This technique becomes simpler with culture methods using pure adult human corneal endothelial cells on a thermo responsive poly-N-isopropylacrylamide-grafted surface. The cells are cultured for 21 days at 37°C where the second passage of the culture was used. Once the culture was confluent with regular phenotype and cell density, the culture temperature was lowered to 20°C to detach the cells as a laminated sheet. Cellular morphology and intracellular connections were studied using ZO-1, while the ionic pumping machinery was studied by the detection of Na+K+-ATPase. The cultured CECs behaved like an in vivo endothelium and the cornea restored normal thickness after transplantation proving the efficiency of bioengineered CECs [63,64]. Lai and colleagues attached monolayer obtained from the culture of CEs on Poly-N-isopropylacrylamide (PNIPAAm), to a gelatin hydrogel discs and transplantated the graft into rabbit eyes.

After an initial edema, these animals recovered corneal transparency within two weeks post transplantation [65].

Another study reported the utilization of fabricated bioengineered CEC sheets. The cells were cultured on type IV-collagen-coated plates and the cells were allowed to expand and were then seeded on the temperature-responsive culture dishes. As above, the cells were harvested without any enzymatic treatment. Similar to the above parameters, cell morphology, density, ion exchange pumps and cell junctions were studied similar to in vivo corneal standards. The transplantation resulted in the functional stromal hydration in vivo [66].

The phenotypic production of human tissue-engineered corneal reconstruction using all three corneal cell types has also been described. A stromal tissue was reconstructed after secreting the fibroblasts that were cultured in serum and ascorbic acid based medium to form a matrix. The endothelium and epithelium was seeded on either side of the stroma using air-liquid interface. Further, the physiologic and phenotypic characteristics were studied. It was seen that the hemi desmosomes and a membrane rich in laminin V and collagen VII was secreted by basal epithelial cells. Endothelial cells showed functional properties as described in the previous paragraphs. Fibroblasts that is untransformed along with epithelium and endothelium with no need for exogenous biomaterial is a creative approach to produce a complete tissue-engineered cornea [67].

Corneal endothelium constructed on a devitalized carrier showed functional outcome when tissues were transplanted in vivo of a feline model. The allogenic feline corneal endothelium were seeded on a devitalized human cornea and cultured for 2 weeks. Slit lamp examination was carried out daily up to 7 days until euthanization on autologous, allogenic or human xenogenic native corneas. Nine corneas out of eleven reconstructed grafts and all the native controls were clear and the controls remained thick and opaque without endothelium. The physiologic functions of the cells were checked, even if this method showed a short term (7 days) functional success. Devitalized stroma has advantages including progressive repopulation of the donor stroma by the recipient's keratocytes, high optical quality, elimination of donor keratocytes, reduction of antigenicity and attachment of endothelium with restoration of complete functionality that indicate high biocompatibility, which is a limitation with synthetic corneal substitutes [68].

Another study showed a preparation method of matrices from bovine corneal endothelial cells. The primary cultures were maintained in humidity with Minimum Essential Medium (MEM) and iron-supplementation calf serum. After confluence, cell stripping was performed using 0.5% sodium deoxycholate (detergent treatment) for 5 minutes at Room Temperature (RT) followed by washing with Earle's Balanced Salt Solution (EBSS) and EDTA. The matrices were preserved for a month at hypothermia in EBSS and were used for culturing. Trypsin treatment was performed on the endothelial cell surface and then incubated for half an hour at RT. The endothelium was scrapped off and the trypsin was inactivated using 10% serum based medium. Cells were then centrifuged and resuspended in the human corneal endothelial basal medium containing sodium selenite in matrix coated culture dishes incubated in humidity, 5% CO2 and 37°C. After 2-4 weeks, the confluent cells were treated with trypsin and the detached cells were plated again at 1:2 to 1:4 ratio in matrix-coated plates. Sub-cultured cells were fed with CEC basal medium or on plastic coated dishes of bovine fibronectin in serum-free MEM. However, the endothelial cell adhesion and proliferation has been tested on extracellular matrices. CECs were

plated on purified fibronectin or matrices on tissue culture dishes cultured with bovine corneal endothelial cells. Fibronectin showed good attachment with cells, but proliferation was slow. However, cells prepared on matrices with bovine corneal endothelium were allowed to grow to fill the flask. Karyotyping showed no presence of cells from bovine origin [18].

Thus, many scaffolds have been described in the literature so far having proven efficiency *in vitro* or in animal models. The scaffold material can be biologic or synthetic, permanent or biodegradable. Common biological materials in scaffolds are collagen, fibronectin, and hyaluronan. However, the next step forward would be to check the feasibility of the use of these scaffolds *in vivo*.

Future Directions

Human corneal endothelium is a physiologically important monolayer of cells, with a simple, but crucial, role to maintain corneal clarity. In order to keep the entire cornea transparent, it is essential for corneal endothelium to retain the unique monolayer, through which the tissue controls active pump and barrier functions. Decompensation of the corneal endothelium resulting from various causes ultimately leads to deficiency in pumping fluids out of the stroma, leading to corneal edema, loss of visual acuity, and cornea-related blindness. The function of the endothelium is compromised if the cell density falls below a critical threshold of 500 cells/mm². Various methods have been attempted to treat endothelial dysfunctions, and in recent years, DSAEK and DMEK have been extensively employed. However, these relatively new procedures still face some obstacles, such as the worldwide shortage of transplantable donor corneas, continuing cell loss after transplantation, technical difficulty, and primary graft failure. In order to address the worldwide shortage of donor corneas, the idea of one donor cornea for each patient has been challenged, and the concept of using one donor cornea for treating multiple patients has yet to be widely accepted. Such a timely goal prompts researchers to establish optimum technologies for isolation and cultivation of CECs. Testing several cultivation methods and scaffolds will eventually lead to better transplantation outcomes. With mark of stem cells and regenerative medicine, the next ambitious goal would be the identification and amplification of corneal endothelial stem cells to treat endothelial disorders reducing invasiveness of clinical interventions. Although, maintenance of the undifferentiated stem cell nature, promotion, amplification, molecular mechanisms and differentiation are still the key issues to learn, with the on-going studies, cultured endothelial cells for transplantation in humans seems to be a possible option in the near future.

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