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Review

Characterization and Prospective of Human Corneal Endothelial Progenitors

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Abstract

Corneal endothelial cells play a critical role in maintaining corneal transparency and dysfunction of these cells caused by aging, diseases (such as Fuch's dystrophy), injury or surgical trauma, which can lead to corneal edema and blindness. Due to their limited proliferative capacity *in vivo*, the only treatment method is via transplantation of a cadaver donor cornea. However, there is a severe global shortage of donor corneas. To circumvent such issues, tissue engineering of corneal tissue is a viable option thanks to the recent discoveries in this field. In this review, we summarize the recent advances in reprogramming adult human corneal endothelial cells into their progenitor status, the expansion methods and characteristics of human corneal endothelial progenitors, and their potential clinical applications as corneal endothelial cell grafts.

Key words: Cornea, Endothelial, Progenitors, Tissue Engineering.

Introduction

The human corneal tissue is composed of different layers including a stratified epithelium, Bowman's layer, stroma, Descemet's membrane, and endothelium at the inner surface. The endothelial cells originate from cranial neural crest cells, forming a single monolayer of hexagonal cells lining the Descemet's membrane of the posterior cornea [1], and play a critical role in mediating vision function [2]. For example, corneal endothelium maintains the corneal transparency, stromal hydration and vision by mediating hydration (termed pump function) and preventing aqueous fluid from entering the stroma while also allowing permeability of nutrients (termed barrier function) (reviewed in [2, 3]). Unlike other species, human corneal endothelial cells (HCEC) are notorious for their limited proliferative capacity *in vivo* [4] due the mitotic block at the G1 phase in the cell cycle [5]. Hence if the endothelium were to become injured or become dysfunctional, there would be no proliferation to compensate for the cell loss and corneal blindness may occur [6, 7]. Until now, the only effective medical treatment is corneal transplantation from healthy donor cadavers. However due to the increasing aging population globally [8], there is an increasingly shortage of donor supply. Thus, it becomes necessary to seek alternative treatment options and one such promising therapeutic modality is the successful engineering of HCEC surgical grafts. In this review, we will discuss the current knowledge of adult corneal endothelial stem cells or progenitors with limited differentiation potential, the engineering of such HCEC grafts, and the potential application of

HCEC tissue engineering.

Difference between ESC, iPSC and Adult Progenitors

Stem cells include embryonic stem cells (ESC), induced pluripotent stem cells (iPSC) and adult stem cells (progenitors). Although ESC have unlimited capacity for self-renewal and powerful pluripotency to differentiate into any type of cells in the human body theoretically, immune-rejection, teratoma formation, induction uncertainty and ethical concerns have hampered their progress towards any clinical applications. Since discovery of iPSC [9], a number of advantages have been proposed, such as a possible autologous approach to circumvent problems of immune-rejection and ethical concerns. Nevertheless, serious safety problems have been raised over the use of retroviral or lentiviral vectors in the creation of iPSC, which may induce genomic alteration and carcinogenesis [10-12]. In addition, their differentiation potential is uncertain. On the other hand, adult progenitors or somatic stem cells have limited differentiation potential, are located in a number of adult tissues such as the bone marrow, brain, heart, limbus, skeletal muscle and skin (reviewed in [13]) and their use avoids the ethical and differentiation potential concerns [14-17]. However, as such the case for all the aforementioned cells, progenitor stem cells are not easy to isolate, expand, and be maintained.

Origination of Human Corneal Endothelial Progenitors (HCEP)

Human corneal endothelial cells have been discovered and characterized over the last few decades [18-22]. In 1982, a group of so-called Schwalbe's line cells were first reported by Raviola et al [23]. These cells are located beneath the Schwalbe's ring, forming a discontinuous cord in the transition region of the anterior part of the trabecular meshwork and the corneal endothelium [23]. After laser trabeculoplasty, the proliferation of these cells was noted, suggesting that Schwalbe's line cells may have progenitor cell-like properties [23]. A similar observation was also reported in human laser-treated explants [24]. In another study, functional corneal endothelial cells were generated from these progenitors with a high proliferative potential and lineage [25].

It was found that HCEC from the corneal periphery and not the central area proliferated suggesting the presence of progenitor cells only in the peripheral area of the cornea (unpublished data). These results are consistent with the observance of telomerase activity in the peripheral and middle corneal areas, but not in the central cornea [26-28]. In the past decade, these results have been confirmed by a number of published articles that have suggested that endothelial progenitors are in fact present in the human cornea (reviewed in [29]). However, no specific markers have been used to identify endothelial progenitors *in vivo*. Therefore, the origin of the endothelial progenitors still cannot be clearly defined.

Expansion of HCEP

A scraping method was first used to isolate HCEC from the cornea and unlock their mitotic block *ex vivo* [30, 31]. Explant culture has been used for the expansion of HCEC up to 6 months and such expansion may produce small, hexagonal cells [32]. It has been found the sphere number were much higher in the peripheral area than that in the central area of the cornea, indicating a higher rate of self-renewal capability from the cells in the peripheral area [33]. Corneal endothelial aggregates (spheres) express a number of neural crest markers and may differentiate into various neuronal lineages [34], which is not surprising considering the corneal endothelium originates from neural crest cells in the embryonic development [25, 35]. HCEC in the spheres are small and hexagonal and are able to expand at a higher density with a higher number of BrdU-positive labeling, suggesting that HCEC sphere culture contain endothelial progenitors [34]. In a rabbit model, injection of corneal endothelial progenitor spheres into the eye restored the endothelial function and resulted in decreased corneal edema [36]. In addition, when cultured on denuded human amniotic membranes, these cells show a typical hexagonal shape and healthy tight junctions as determined by immunostaining of ZO-1 [37, 38].

Interestingly, when the cells are incubated in 0.02% EDTA for an hour, expression of neuronal markers is not observed even in the spheres (Reviewed in [39]). In fact, an EDTA/trypsin method has been developed to unlock the mitotic block of *in vitro* HCEC by dissociating their intercellular junctions and perturbing contact inhibition [20, 40], then culturing resultant single cells in bFGF- and serum-containing media [19, 20, 25, 40-45]. However, these conventional approaches can potentially trigger endothelial-mesenchymal transition (EMT), leading to the loss of the HCEC phenotype [7, 46], and loss of their progenitor status [47]. Such change of phenotype is due to activation of canonical Wnt signaling in the presence of EGF and/or bFGF, and even more-so when TGF-β1 is added, which activates canonical TGF-β signaling resulting in nuclear translocation of

pSmad2/3 and Zeb1/2 [48]. Interestingly, the use of SB431542, a selective inhibitor of the TGF-β receptor, may block EMT in HCEC [49]. With this in mind, the blockade of canonical Wnt-Smad2/3-Zeb1/2 signaling is necessary during the expansion of HCEC.

In contrast to EDTA/trypsin, collagenase removes interstitial but not basement membrane of the corneal tissue [20]. Such resulting aggregates can be expanded effectively in a medium containing LIF and bFGF [35, 47]. LIF has been shown to delay contact-inhibition and is significantly more effective in promoting HCEP growth with bFGF [47, 50]. Many substrates for culturing these HCEP and HCEC [18, 22, 40, 41, 51], including artificial matrices, such as collagen I and fibronectin (FNC) [52], chondroitin sulfate and laminin [19], laminin-5 [53], matrigel [51] and FNC coating mix [27]. We have selected Collagen IV as the coating substrate because collagen IV has been identified as a better substrate for expansion of HCEP for tissue engineering purposes [20, 29, 54]. Although there has been reported successful amplification of HCEC [55, 56], up to now, no clinical application of cultured HCEC grafts has been reported.

Characterization of HCEP

A distinct subpopulation of cultured corneal endothelial cells has been discovered, showing colony-like structures with small size [57]. These cells are heterogeneous, have characteristic sphere growth tendency and plasticity to change to other type of cells, with high proliferative potential, dependent on endogenous upregulation of telomerase [25, 58] (also reviewed in [34]). The corneal endothelial progenitors are characterized as a group of small endothelial cells expressing p75NTR, SOX9, FOXC2, Twist, Snail and Slug with higher proliferative potential [25, 35, 59-63]. In addition, we have characterized human corneal endothelial progenitors as a group of cells expressing a number of ESC markers, such as cMyc, KLF4, Nanog, Nestin, Oct4, Rex1, Sox2, SSEA4 and NC markers such as AP2α, AP2β, FOXD3, HNK1, MSX1, p75NTR and Sox9 [47].

Reprogramming of HCEP as a Novel Strategy of Engineering HCEC

BMP signaling is necessary for programming of ESC to vascular endothelial cells [64, 65], and are important for reprogramming iPSC [66]. Recently, we have also reported that p120-RhoA-ROCK signaling may activate and elicit canonical BMP signaling in the growth of HCEC in MESCM [47] by weekly treatment of p120-Kaiso siRNAs for 5 weeks. Such expansion is associated with translocation of membranous p120 to the nucleus and release of nuclear Kaiso, a transcriptional repressor. That is, contact inhibition of HCEC monolayers can be safely perturbed by transient knockdown with p120 catenin (hereafter p120) ± Kaiso siRNAs to activate p120-Kaiso signaling via eliciting nuclear translocation of membranous p120 and nuclear release of the transcription repressor Kaiso. This then leads to RhoA-ROCK-canonical BMP signaling [47] when cultured in LIF-containing MESCM but non-canonical BMP-NFκB signaling when cultured in EGF-containing SHEM [67]. The former but not the latter also results in significant expansion of HCEC monolayers due to reprogramming into neural crest (NC) progenitors [47].

LIF, a member of the IL-6 family, is a key cytokine for sustaining self-renewal and pluripotency of mouse ESC and iPSC [68-71]). Upon binding to the LIF receptor, LIF activates JAK, which phosphorylates latent STAT3. pSTAT3 dimerizes and enters the nucleus to target expression of KLF4 [72] and Nanog [73]. We have recently reported that LIF-JAK1-STAT3 signaling indeed operates in HCEC monolayers cultured in MESCM [50]. The mechanism for such reprogramming is activation of the autoregulatory network of Oct4-Sox2-Nanog and miR-302 cluster in promoting self-renewal and pluripotency [67]. In this process, nucleus-translocated Oct4, Sox2, and Nanog directly binds to the promoter to activate expression of this miR-302 cluster [74, 75], and miR-302 then indirectly induces expression of Oct4, Sox2, and Nanog by reducing the expression of developmental genes [76, 77]. This approach is justified by our recent report showing that the reprogramming of NC progenitors also involves overexpression of miR 302b/c, which is completely blocked by RhoA inhibitor CT-04, ROCK1/2 siRNAs and BMP inhibitor Noggin [47], suggesting the overexpression of miR 302b/c is mediated by RhoA-ROCK1/2 signaling. This reprogramming resembles what has previously been reported [78, 79] that forced expression of transcription factors, e.g., Oct4, Sox2, KLF4 and c-Myc (SKOM), is a novel strategy [80] to reprogram somatic cells to iPSC [81, 82].

Barrier for Reprogramming HCEP

In mammalian cells, the G1/S transition is blocked in "contact inhibition" but facilitated in proliferation by E2F, of which the activity is inhibited by non-phosphorylated retinoblastoma tumor suppressor (Rb) [83]. Release of inhibition mediated by phosphorylation of Rb is controlled positively by cyclin D1/cyclin-dependent kinase-4 (CDK4) and cyclin E/CDK2 complex, but negatively by cyclin-dependent kinase inhibitors (CKIs) such as $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, $p19^{INK4d}$, $p21^{CIP1}$, $p27^{KIP1}$,

and $p57^{KIP2}$ [84]. Without p120-Kaiso knockdown, we have recently reported that LIF-JAK1-STAT3 signaling delays contact inhibition [50]. MESCM without LIF, but not bFGF, delays contact inhibition by preventing nuclear translocation of $p16^{INK4a}$, a process blocked by STAT3 siRNA [50].

Bmi-1, a member of the Polycomb Group (PcG) gene family of proteins that function as chromatin modifiers, is a suppressor of the Ink4a locus including $p16^{INK4a}$ [85-87]. $p16^{INK4a}$ belongs to the family of cyclin-dependent kinase inhibitors involved in cell cycle arrest at the G1 phase [88]. Nuclear $p16^{INK4a}$ is a hallmark of contact inhibition because $p16^{INK4a}$ binds to CDK4/6 inhibiting its kinase activity thereby preventing Rb phosphorylation during G1 to S transition [reviewed in [89]]. Hence $p16^{INK4a}$ controls HCEC senescence [77] and reprogramming [88, 90, 91]. p120-Kaiso knockdown releases nuclear Kaiso to the cytoplasm [46, 47], and activates both Rb and $p16^{INK4a}$ (reviewed in [92]), thus it is speculated that the mitotic block mediated by $p16^{INK4a}$ facilitates contact inhibition and senescence as a barrier against reprogramming and that such a barrier can be overcome by nuclear translocation of pBmi-1 facilitated by both STAT3 signaling and nuclear release of Kaiso. The aforementioned delay in contact inhibition may also be achieved by transit activation of LIF-JAK1-STAT3 signaling that also delays eventual nuclear translocation of $p16^{INK4a}$ [50]. Thus, JAK2-STAT3-Bmi-1 signaling is another downstream signaling of p120-Kaiso-RhoA-ROCK signaling that participates in reprogramming of HCEC into progenitors via inhibition of $p16^{INK4a}$-mediated senescence [93].

Potential Clinical Application of Human Corneal Endothelial Grafts after Preclinical Animal Studies

Pre-clinical animal studies are the required method for examination of the safety and efficacy of human corneal endothelial grafts, including those expanded from HCEP. Because Descemet's stripping automated endothelial keratoplasty (DSAEK) and Descemet's membrane endothelial keratoplasty (DMEK) has become a standard procedure for corneal transplantation in patients with endothelial dysfunction in the last decades [94-97], transplanting only the HCEC sheets has become a standard procedure for treatment of CEC dysfunction [42]. In 2001, primary cultured HCEC were constructed onto the denuded Descemet's membrane for a test transplantation of ex vivo human corneal endothelium [40]. In this case, the recipient cornea was cultivated in organ culture for up to 2 weeks. The mean endothelial cell density in the transplanted corneas was 1895 cells/mm^2 (1503–2159 cells/mm^2), and was deemed a success [40]. Amniotic membrane has also been introduced as a reliable carrier for cultured HCEC transplantation [41]. The density of the HCEC on the amniotic membrane was shown to be greater than 3000 cells/mm^2, similar to that of *in vivo* density. Another potential carrier is collagen I which has been successfully used for cultured monkey corneal endothelial sheets [98, 99] and we are currently testing in a mini-pig model with cultured endothelial grafts.

The current limitations and challenges for the research of HCEP are there are many difficulties for isolation and expansion of a population of HCEP without contamination of other type of cells and without change of the cell phenotype as *in vitro* culture time passes by. Therefore, there is no cell-based therapies for cure of human corneal endothelial dysfunction so far. However, the research in this field has progressed rapidly. Hopefully, we will resolve those issues in the near future.

Conclusions

This review has highlighted the latest discoveries and innovations in corneal endothelial engineering. The novel techniques presented here demonstrate the potential future treatments of CEC dysfunction.

Abbreviations

bFGF: Basic fibroblast growth factor; Bmi-1: B lymphoma Mo-MLV insertion region 1 homolog; BMP: Bone morphogenic protein; BrdU: Bromodeoxyuridine; EDTA: Ethylenediaminetetraacetic acid; DMEK: Descemet's membrane endothelial keratoplasty; DSAEK: Descemet's stripping automated endothelial keratoplasty; EMT: Endothelial-mesenchymal transition; EGF: Epidermal growth factor; ESC: embryonic stem cell; FNC: fibronectin; HCEC: human corneal endothelial cell; HCEP: human corneal endothelial progenitor; IL: interleukin; iPSC: induced pluripotent stem cell; JAK: Janus kinase; KLF4: Kruppel-like factor 4; LIF: Leukemia inhibitory factor; MESCM: modified embryonic stem cell medium; NC: neural crest; NFkB: nuclear factor kappa-light-chain-enhancer of activated B cells; NGF: neural growth factor; $p16^{INK4a}$: a tumor suppressor protein functions as an inhibitor of CDK4 and CDK6, the D-type cyclin-dependent kinases that initiate the phosphorylation of the retinoblastoma tumor suppressor protein; p120: p120 catenin; PcG: polycomb group; Rb: retinoblastoma; PKP: penetrating keratoplasty; Rho: Ras homolog gene family; ROCK: Rho-associated protein kinase; siRNA: Small interfering ribonucleic acid; SHEM:

supplemental hormonal epithelial medium; STAT: signal transducer and activator of transcription; TGF: Transforming growth factor; ZO-1: Zona occludens protein 1

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Authors Contributions

Yongsong Liu, Hong Sun, Ping Guo, Min Hu, Yuan Zhang, Sean Tighe and Shuangling Chen contributed to collection of information, organization and part of writings. Ping Guo and Yingting Zhu oversaw this project and finalized this review.

Competing Interests

The authors have declared that no competing interest exists.

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