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Review

Corneal Endothelial Cells Over the Past Decade: Are We Missing the Mark(er)?

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Corneal endothelial dysfunction is one of the leading causes of corneal edema and visual impairment, requiring corneal endothelial transplantation. The treatments are limited, however, by both logistics and a global donor shortage. As a result, corneal researchers are striving to develop tissue-engineered constructs as an alternative. Recently, the clinical results of the first patients treated using a novel corneal endothelial cell therapy were reported, and it is likely many more will follow shortly. As we move from lab to clinic, it is crucial that we establish accurate and robust methods of proving the cellular identity of these products, both in genotype and phenotype.

In this review, we summarized all of the markers and techniques that have been reported during the development of corneal endothelial cell therapies over the past decade. The results show the most frequently used markers were very general, namely Na^+/K^+ ATPase and zonula occludens-1 (ZO-1). While these markers are expressed in nearly every epithelial cell, it is the hexagonal morphology that points to cells being corneal endothelium in nature. Only 11% of articles aimed at discovering novel markers, while 30% were already developing cell therapies. Finally, we discuss the potential of functional testing of cell products to demonstrate potency in parallel with identity markers.

With this review, we would like to highlight that, while this is an exciting era in corneal endothelial cell therapies, there is still no accepted consensus on a unique endothelial marker panel. We must ask the question of whether or not we are getting ahead of ourselves and whether we need to refocus on basic science rather than enter clinics prematurely.

Introduction

The corneal endothelium is a single layer of cells that covers the posterior cornea and is organized in a characteristic honeycomb pattern. Human corneal endothelial cells (HCEnCs) are primarily responsible for regulating stromal hydration, which is inherently related to the transparency of the tissue.[1] The corneal stroma makes up 90% of corneal thickness and is composed of collagen fibrils, with a uniform diameter of 31 nm, that cannot lie closer than 62 nm to each other. It is this high degree of organization as well as the avascularity and relative state of dehydration that is responsible for the transparency of the healthy cornea.[2]

Because the cornea is predominantly avascular, it depends on passive oxygen and nutrient flow, both from the precorneal tear film and from the anterior chamber aqueous fluid. Nutrients from the aqueous fluid pass through the corneal endothelium, which acts as a leaky barrier (Fig. 1).[3,4] An unopposed constant fluid influx into the cornea, such as occurs in severe endothelial dysfunction, results in corneal

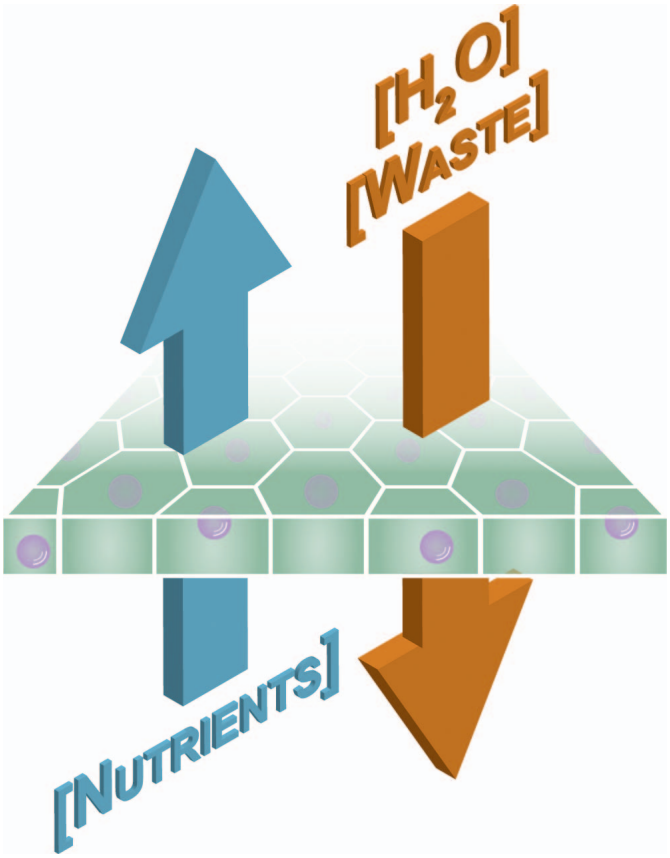

Figure 1. The corneal endothelium leaks anterior chamber fluid to the corneal stroma and pumps back H_2O and metabolites to prevent corneal edema.

edema as the accumulated fluid distorts stromal interfibrillary space. The disorganized lamellar arrangement seen in corneal edema results in increased backscatter of incoming light, leading to loss of corneal transparency and thus visual impairment.[2] Hence, one of the main functions of the corneal endothelium is to act as a pump, creating ion gradients to counteract this flow and maintain an optimal stromal hydration. The balance between passive diffusion and active pumping is known as the "pump-and-leak mechanism" and is indispensable for corneal transparency.[3]

HCEnCs are not thought to undergo cell division in vivo, although they can enlarge and migrate to cover a defect, thereby maintaining the cell layer's net pump function. When extensive damage to the corneal endothelium occurs, by trauma, infection, inflammation, or inherited disease, the result is a low cell density (under approximately 500 cells/mm^2); the net pumping capacity can no longer compensate for the passive leakage and edema develops.[5] At present, the only option for patients suffering from this form of corneal decompensation is surgical replacement of the endothelium by endothelial keratoplasty (EK).

In 1905, Eduard Zirm performed the first successful human corneal transplantation or full-thickness penetrating keratoplasty (PK).[6] This landmark surgery paved the way for modern keratoplasty, currently the most successful solid-organ transplantation.[7] Modern corneal transplantation favors a more selective approach over full-thickness transplantation, which has resulted in improved recovery times and reduced risks.[8] In 2012, EK overtook PK as the most frequently performed transplantation technique for endothelial disease, and the numbers are continuing to rise.[9,10] Corneal endothelial dysfunction accounts for approximately 40% of all corneal transplantations performed at present.[10] Unfortunately, the current one-to-one use of cadaveric donor tissue is extending the existing waiting lists every day.[11] In an effort to address this, researchers have begun investigating tissue-engineering approaches to develop suitable alternatives.

The idea of growing corneal endothelial cells on scaffolds to make multiple transplants is not new. It was first proposed in 1978, and despite it being the most explored strategy, it has not yet progressed to routine use in the clinic.[12] Recently, Kinoshita et al.[13] performed a phase I clinical trial based on the injection of an ex vivo expanded cell suspension. The expanded cells were injected into the anterior chamber, and the patient was positioned face down so that the cells could attach.

Despite the intrinsic homologous use of the corneal endothelial cells, endothelial cell therapy is classified as a tissue-engineered product rather than as a form of transplantation. This is because the cells are regarded as being extensively manipulated.[14,15] Bioengineered tissue products are subject to a higher level of regulatory control than are standard therapies and must meet extremely high levels of quality assurance. As a result, these products can take years to make it to the market. In Europe they are regulated by the European Medicine Agency (EMA) and in the United States by the Food and Drug Administration (FDA). Acts that are regarded as "extensive manipulation" include enzymatic digestion of the donor tissue and cell (sub)culturing and any other acts that could induce changes in the surface protein composition or in gene expression.[16] Proving that a cell product is exactly what it purports to be is one of the more challenging aspects of securing regulatory approval. Accurate genotype and phenotype markers are key.

Inside an eukaryotic cell, DNA is transcribed to an RNA strand that is then further translated to

proteins.[17] The makeup of a cell is therefore dictated by the genetic material within a cell, called the genotype, which gives rise to a specific subset of proteins. The phenotype, on the other hand, can be described as the specific set of expressed amino acids, peptides, or proteins that make up a unique fingerprint of a cell at a certain moment in time. So while the phenotype of a cell is determined predominantly by the genotype, it is also further influenced by epigenetics and environmental factors. Although they are inherently related, the correlation of transcription-to-translation is not one to one, but rather is regulated by a variety of pre- and posttranslational mechanisms such as transcription factors, chromatin packing, and noncoding RNA strands, which can result in different protein-to-mRNA ratios for different genes.[18] It is important to study both a cell's genotype and phenotype, particularly in a cell therapy manufacturing environment, to mitigate potential risks to the patient.

A newly re-formed endothelial cell layer in vivo should function as both a leaky barrier and an actively pumping cell layer to preserve the cornea's ideal hydration status. Proper cell function must be validated before heading to in vivo preclinical or clinical trials. Specifically, the proper characterization of a cell product must be validated, which includes guaranteeing the cells' identity, purity or impurity, potency, and tumorigenicity by following the EMA and FDA regulatory guidelines.[19] In this review, we aimed to review the state of the art of endothelial cell identity and potency characterization with a complete literature overview of in vitro characterization over the past 10 years. We included only papers using primary cultured HCEnCs, as it has been shown that microRNA (miRNA) and mRNA expression differs between primary cultures and ex vivo endothelial tissue.[20]

Materials and Methods

Literature Search

The most recent literature search was performed in June 2018 using "corneal endothelium" and "corneal endothelial" as search terms in the PubMed database. The query was further refined to search for only those articles published in the last decade. We then excluded foreign languages (i.e., Japanese and Chinese) and studies other than original research concerning cultured HCEnCs (cHCEnCs), for example, non-corneal neural crest-derived (progenitor) cells. The remaining publications were analyzed and techniques applied, and markers used were extracted. Finally, we report only genotypic and phenotypic markers that were cited more than once (Supplementary Table S1). We clustered every gene-based assay under the collective term "genotype," including PCR and microarrays; we also grouped immunocytochemistry, Western blot, and flow cytometry to discuss the "phenotype." In order to avoid any potential bias, we neither graded the expression patterns nor the quality of the images, but we included the marker on the basis of it being mentioned in the methodology or results section.

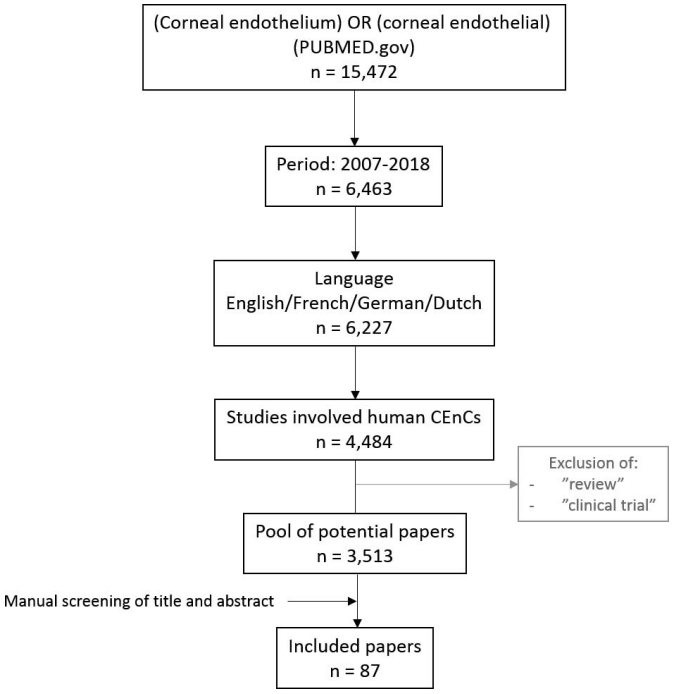

Figure 2. The search strategy that was used to obtain the included papers.

Results

Literature Search

Figure 2 displays the search strategy used for including all relevant papers. The total number of papers found with the query "corneal endothelium OR corneal endothelial" was 15,472. Our search was further narrowed by limiting the included papers to 2007–2018 in English, French, German, and Dutch, which totaled 6227 papers. Finally, we only limited ourselves to experimental studies that involved HCEnCs and ended with a pool of 3513 papers.

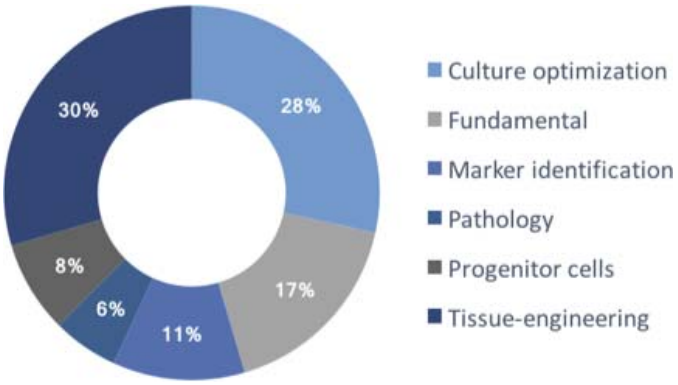

Figure 3. Classification of the included papers.

After manually screening these papers, we obtained 87 papers that reported on genotyping or phenotyping of HCEnCs (Supplementary Table S1).

Classification of the Markers

We classified the articles into six categories according to their content (Fig. 3). Most articles aimed at tissue-engineering corneal endothelial grafts (30%) and at optimizing culture conditions for primary HCEnCs (28%). Publications that focused on finding new markers for primary cells comprised 11% of the work published in the last decade. The other categories, such as investigations in fundamental endothelial cell processes, progenitor cells, and pathology accounted for the final third of the publications, subdivided into 17%, 8%, and 6%, respectively.

The Endothelial Genotype

Reverse transcriptase PCR (RT-PCR) is widely used to study the genotype of cells and is based on measuring mRNA expression that is compared to a set of housekeeping genes to provide a base level of gene expression. The comparison to such genes renders a relative readout of gene expression specific for cells in a certain environment.

Taken together, 33% ($n = 29/87$) of the included studies performed at least one genotypic assay on primary HCEnCs to investigate the expression of 86 different genes in total, with 20 genes that were studied cited at least twice (Fig. 4). The most frequently studied genes in primary HCEnC are Na^+/K^+ ATPase (*ATPA1*; 16%), ZO-1 (*TJP1*; 11%), and collagen type VIII (*COL8*; 9%). While RT-PCR was the most preferred method, other sporadic techniques include microarrays, next generation sequencing, and phage display.[21–23]

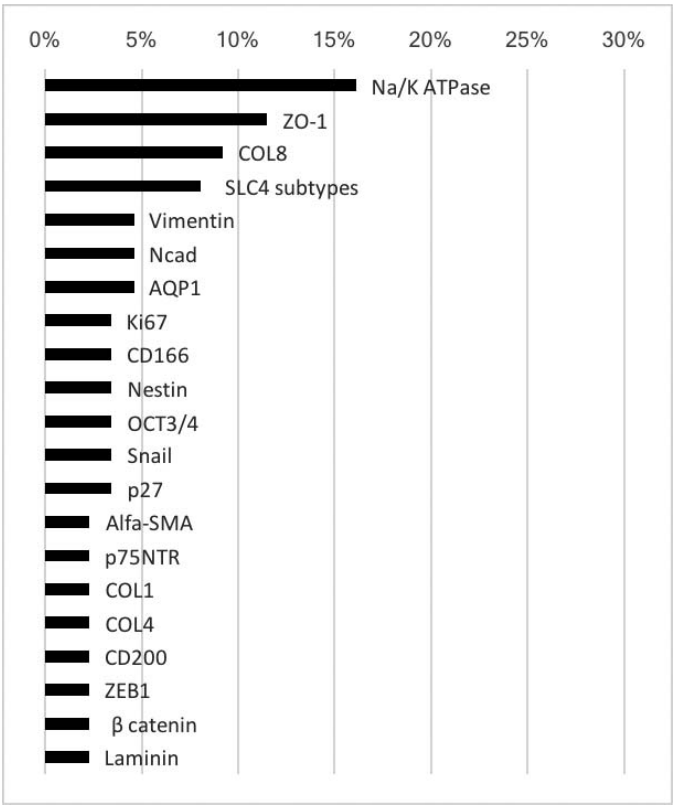

Figure 4. Ranking the cited genes that have been used for cHCEnC identification.

The Endothelial Phenotype

The most common way to show the proper cell phenotype was by antibody-based assays, predominantly immunocytochemistry, flow cytometry, and Western blot. Phenotypic assays were performed at least once in 93% of the included articles. After analysis, we could discriminate 87 different phenotypic markers. Figure 5 shows the relative frequency of markers used that have been cited at least two times ($n = 48$). The most frequently used marker was ZO-1, which was reported in 75% of publications. The second and third most frequently cited markers were Na^+/K^+ ATPase and Ki67, occurring in 53% and 17% of the cases, respectively. ZO-1 and Na^+/K^+ ATPase were both used in 52% of the phenotypic studies. Immunocytochemistry was by far the most frequently used technique, appearing in 90% of the studies, followed by Western blot in 16% and flow cytometry in 9%.

A Closer Look at the Two Most Cited Markers

ZO-1 Protein

Cells are connected through intercellular contacts known as the junctional complex, which is an

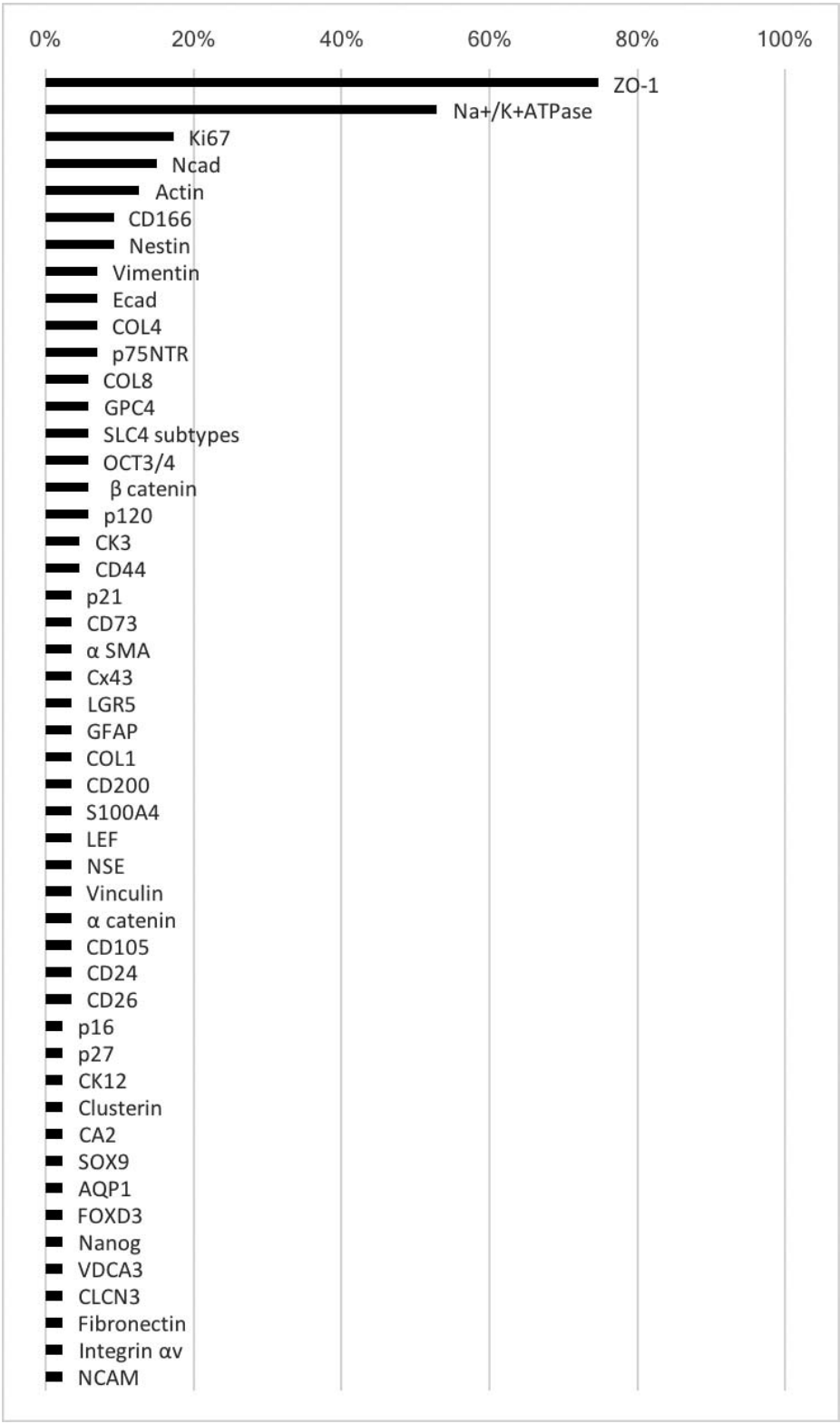

Figure 5. The prevalence of phenotypic markers cited in the past decade.

accumulation of cell type–dependent proteins that make up anchoring, communication, and tight junctions. The latter, also called zonula occludens, is a sealing plaque at the most apical side of the junctional complex; it is typically abundant in epithelia and endothelia.[24,25] Tight junctions act mainly to regulate the paracellular leakage of ions and solutes, preventing the intermixing of basolateral and apical molecules in the process and thereby maintaining cell polarity.[24] ZO-1 proteins, encoded by the *TJP1* gene, are scaffolding proteins and regarded as an integral part of these tight junctions; they are generally expressed in every epithelial cell layer in the human body.[26]

ZO-1 expression in endothelium is ascribed to its function as a leaky barrier for corneal endothelial cells particularly.[24] The specific expression pattern reveals a belt of tight junctions that delineates the hexagonal shape of HCEnCs and is held responsible for the passive diffusion of nutrients from the anterior chamber to the cornea.[27] A hexagonal or honeycomb ZO-1 expression is, however, not exclusive to the corneal endothelium as a very similar pattern can be seen in retinal pigment epithelial (RPE) cells; thus, while it is quite typical, it is not a perfect biomarker.

Na^+/K^+ ATPase

Nonexcitable eukaryotic cells display a membrane potential which is essential in driving essential cell functions and requires both a low cytoplasmic concentration of Na^+ and Ca^{2+} and a high concentration of K^+ ions.[28] As the extracellular milieu contains opposing concentrations, cells need to be able to transport Na^+ ions out and K^+ ions in against their electrochemical gradient to maintain the proper membrane potential. This active process requires ATP hydrolysis for energy and is crucial to maintaining the proper membrane potential. Na^+/K^+ ATPase is expressed virtually in every cell due to its conserved role in cell homeostasis in eukaryotes.

In corneal endothelial cells, Na^+/K^+ ATPase also takes part, together with all other ion channels, to establish a membrane potential of around −30 mV. However, it is hypothesized that they take part in the creation of an additional local hyperosmotic gradient to enable a fluid flow from the stroma toward the anterior chamber. The ion pumps' crucial role in this process is widely accepted, but the exact mechanism has not been clarified to date.[29] Similar to ZO-1, the hexagonal staining pattern is typical, but unique, and it is also seen in RPE cells.

The First Panel of Clinical Markers

Recently, Kinoshita et al.[13] have made a very significant contribution to advancing the clinical translation of homogeneously cultured corneal endothelial cells. The group discriminated cell therapy–grade HCEnCs from cells that either undergo endothelial-to-mesenchymal transition or become senescent by analyzing the gene and miRNA expression of different subpopulations in vitro.[20] More specifically, they reported that endothelial cells displaying $CD44^-CD166^+CD133^-CD105^-CD24^-CD26^-$ expression exhibit the correct genotype and phenotype for use in cell therapy.[30] The absence of CD44, CD24, and CD26 renders cells without aneuploidy, thereby relating phenotypic analysis to the cellular karyotype.[31] Furthermore, miR34a detection in culture medium was linked to a lack of CD44 expression and thus to the absence of aneuploidy.[32] Although miRNA analysis shows some potential for use in the validation of cell therapy products, we are concerned about the concomitant isolation of miRNAs from the fetal bovine serum and mesenchymal stem cell–conditioned medium that are used as additives in their cell culture medium.[33,34]

Evaluation as an Endothelial Tissue

Apart from the aforementioned assays that focus on cellular identity, we should also consider the potency of an endothelial cell therapy, that is, validating a laboratory-grown endothelium as a functional tissue. Once again, such assays are based on the pumping property of the endothelium, but in a quantitative manner. The most prevalent method reported in the literature, which is applied not just to human endothelium, is the Ussing chamber.[35–38] The electrical potential is measured over a cell layer using this technique, which provides information about the pumping function and the "tightness" of the tissue-engineered endothelium. Another sporadic attempt to functionally qualify an endothelium includes measuring the capacity to dehydrate a corneal stromal equivalent made of collagen, thereby simulating the endothelium's deswelling capacity in vivo.[39]

Discussion

It is striking that the combination of markers, that are currently used to identify healthy corneal endothelial cells preclinically are not particularly unique to these cells or even to their lineage. The frequent use of these markers is most likely attributable to the lack of

a better, more specific alternative. In examining the published research, we noted that there is a significant issue raised by the methodology in the majority of these studies: namely, an overreliance on immunocytochemistry. This may be because of the difficulty in expanding corneal endothelial cells in the quantities needed for other methods, such as Western blot, flow cytometry, and PCR assays. A positive signal in immunocytochemistry alone is not uniformly conclusive though. The pattern of staining, subcellular localization, or degree of expression is hardly meaningful, considering that a marker such as Na^+/K^+ ATPase is expressed by all eukaryotic cells, so its mere presence cannot be considered to be sufficiently specific.

Attempts at finding unique corneal endothelial markers have been made using techniques such as phage display, microarray analysis, and next generation sequencing, but this contributes to only 11% of the research published in the last decade.[18,28–30] The unique phenotypic markers that have reportedly been found to be linked to healthy HCEnCs in these studies are CD166, CD200, GPC4, HLA-ABC, and PD-L1.[21,32,40,41] In addition to markers that positively identify HCEnCs, attention should also be paid to the absence of certain gene or protein expressions, in particular α-SMA, CD9, CD24, CD26, CD44, CD73, CD90, CD105, and CD133 for phenotypic studies, and Snail, ZEB1, and vimentin, which have been shown to be negative in HCEnCs.[19,32–35] Nevertheless, these markers are challenged (i.e., CD166[20]) and have not been widely adopted by other groups to date.

Additionally, the EMA guideline indicates that morphologic analysis for adherent cells could also be a helpful tool for identification. Corneal endothelial cells are most frequently introduced as hexagonal in shape; however, only a few publications perform actual morphometric analysis. Peh et al.[42] used a circularity index to grade the shape of HCEnCs in order to discriminate them from elongated fibroblasts, though we must also bear in mind that hexagonality per se is not a unique feature in human ocular cells.

Conclusion

It is clear that there is not yet a widely accepted consensus about a correct phenotypic and genotypic expression profile for healthy corneal endothelial cells. At both the gene and protein levels, the most frequently used markers are Na^+/K^+ ATPase and ZO-1, which are ubiquitously expressed and therefore are not conclusive enough to consistently guarantee corneal endothelial cells for cell therapy. Recently, a pragmatic approach to the qualification of HCEnCs was used by Kinoshita et al.,[10] which was then applied to produce an endothelial cell suspension for injection in human patients.

Time will tell if this will become the gold standard. We believe, however, that additional functional testing of the endothelium as a tissue should play a more prominent and complementary role in the future in order to ensure a good tissue-engineered cell product without relying too much on whether or not proposed markers effectively reflect functional cells.

We have already entered a groundbreaking new era with the surpassing of a crucial milestone, that is, treating the first pioneering patients with cultured HCEnCs. In this review, we would like to emphasize the need for further fundamental research to the process of optimizing corneal endothelial cell therapy.

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