• Review •

Induced pluripotent stem cells as a potential therapeutic source for corneal epithelial stem cells

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Abstract

• Corneal blindness caused by limbal stem cell deficiency (LSCD) is one of the most common debilitating eye disorders. Thus far, the most effective treatment for LSCD is corneal transplantation, which is often hindered by the shortage of donors. Pluripotent stem cell technology including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have opened new avenues for treating this disease. iPSCs-derived corneal epithelial cells provide an autologous and unlimited source of cells for the treatment of LSCD. On the other hand, iPSCs of LSCD patients can be used for iPSCs-corneal disease model and new drug discovery. However, prior to clinical trial, the efficacy and safety of these cells in patients with LSCD should be proved. Here we focused on the current status of iPSCs-derived corneal epithelial cells used for cell therapy as well as for corneal disease modeling. The challenges and potential of iPSCs-derived corneal epithelial cells as a choice for clinical treatment in corneal disease were also discussed.

• KEYWORDS: induced pluripotent stem cells; corneal epithelial cells; limbal stem cell deficiency; disease modeling

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INTRODUCTION

Pluripotent stem cells are primitive cells that are able to be self-renewing, proliferating indefinitely in their undifferentiated state, and differentiate into different cell types[1-2]. They can efficiently differentiate into specific cell types under defined conditions. Therefore, stem cells have been regarded as unlimited source of cell transplantation. Over the last decade, stem or progenitor cells transplantation as a means of replacing tissue have evolved rapidly. There are distinct kinds of stem cells according to their differentiation potential. It has already been found that embryonic stem cells (ESCs) and mesenchymal stem cells (MSCs) can directly differentiate into specialized cells, which attracted considerable interest. However, the future use of these cells is still facing challenges, such as immune rejection and ethical debate for ESCs; the heterogeneity, difficulty of large scale harvest, insufficient source, reduced differentiation capability after multiple generations for MSCs, which limit their clinical application.

The discovery of induced pluripotent stem cells (iPSCs) is considered as a breakthrough. In 2006, Takahashi and Yamanaka[3] reported that mouse fibroblasts could be induced into ESC-like cells and named them "induced pluripotent stem cells". iPSCs have self-renewing and multi-directional differentiation potential, which are similar to ESCs. Differentiated iPSCs generated from the same patient enable their use in autologous transplantation to avoid immune rejection and ethical debate[4]. Moreover cells or tissues for generating iPSCs can be obtained simply and noninvasively. Therefore, iPSCs have drawn particular attention to biological scientists and clinicians[5].

Nowadays the work on iPSCs culture focus on the retinal and retinal cells, which has been revealed a formerly underrated level of intrinsic cellular self-renewing[6]. In recent years there has been considerable progress in the study of iPSCs and corneal diseases. Here we will summarize the recent advancement in iPSCs-derived corneal epithelial cells technology and discuss its therapeutic potential for patients with limbal stem cell deficiency (LSCD). The review will also

highlight the limitations that need to deal with and plausible strategies for the application, the advantage of iPSCs-derived disease models to understand the pathophysiology of cornea disorders, as well as the future of clinical trial for iPSCs as cell sources will also be discussed.

LIMBAL STEM CELLS AND CORNEAL EPITHELIAL CELLS

The development of ocular surface is a dynamic procedure. In the next part of the article, we will discuss the development of ocular surface and particular markers, which make structures specific. The ocular surface is composed of corneal epithelium, conjunctival epithelium and tear film. The surface of the cornea is covered by corneal epithelial cells, which are crucial for maintaining normal eye function. Corneal epithelial cells are derived from the epidermal layer of the embryo. When the human embryo is about 9 mm, the lens bubble differentiates completely from the ectoderm, and then the corneal epithelium differentiates from the ectoderm afterwards. With the continuous development of the embryo, the corneal epithelial cells gradually differentiate and form complete structure[7-8]. The morphology of normal corneal epithelium is like paving stone. Under normal condition the morphological characteristics of corneal epithelium are stable. The dynamic balance of corneal epithelium is important for maintaining normal vision and the integrity of ocular surface. Infection, injury or lack of limbus stem cells can lead to corneal fibrosis[7]. Cell proliferation, morphological changes, and the increased expression of mesenchymal markers of the long-term culture corneal epithelial cells indicated epithelial to mesenchymal transition[9]. The shape of corneal epithelial cells was transformed from cubic into fusiform-like fibroblast. At the same time the connection between epithelial cells was lost[10]. Cytokeratin (CK) 3 and CK 12 were known as specific markers of corneal epithelial cells. It was found that there was connexin 43 in the corneal epithelial cells, which could connect them[11]. When the corneal epithelial cells are in a transient state of proliferation, expression of connexin 43 is negative[12-13]. CK4 and CK13 were markers of non-keratinized epithelium[14]. CK15 was the marker of corneal progenitor cell[15]. In general, connexin 43, CK3, CK12, CK4, CK13 and CK15 were considered as indicator of corneal differentiation[16-17].

The current studies of stem cells related to not only laboratory situations, but also clinical situations. The corneal epithelial cells are constantly shedding or dying due to external environment or internal metabolism. Under the basal cell proliferation and the differentiation of limbal epithelium stem cells (LESCs), which is found in the corneal limbus located between the transparent cornea and opaque conjunctiva, the corneal epithelial cells are contantly repaired by itself[18]. Severe ocular surface disease can damage the limbic region, leading to the reduction or loss of LESCs to some extent and the destruction of barrier structures. That may cause corneal hazing, corneal epithelium defect, or even blindness, which is clinically termed as LSCD[19-20]. Many disease can cause LSCD, like chemical burn or traumatic injury, inherited corneal dystrophy and numerous immune disorders, for example, Stevens-Johnson syndrome[20].

Corneal stem epithelial cells are crucial for repair of corneal surface. They express different markers during the process of proliferation. It is widely believed that the corneal epithelial stem cells belong to unipotent somatic adult stem cells, and the stem cells are exclusively located in a particular limbal structure, which is defined as palisades of Vogt[21]. Limbal epithelial basal layer lacks the expression of keratins CK3 and CK12, the terminal differentiation marker of corneal epithelial cells[22]. The study found that when the central corneal epithelium was damaged, the corneal limbus basal cells were more active than those in the central region of the epithelium. The direction of the cell migration was from the cornea limbus to the central. Hayashi *et al*[23] found that the corneal epithelial stem cells were gradually replaced by LESCs, which located at the base of the cornea limbus. Although it is hard for the corneal epithelial stem cells to be classified and separated by just one particular marker, in recent years, ABCG2 and P63 (ΔNp63α isoform) has been found expressed in LESCs. However, there is no expression of CK3 and CK12 in these cells[24-25]. P63 is a kind of nucleoprotein, which expressed in the limbus in the basal layer of the cornea. The expression of P63 in corneal limbic cells is regarded as a marker of proliferation ability for these cells[26].

The treatment of LSCD includes drug and surgical therapy. Drug therapy such as artificial tear, bandage contact lens and autologous serum eye drop are only applied as adjuvant therapy, which have been only used in very mild cases[27]. Surgical therapy include amniotic membrane transplantation, autologous conjunctival limbal stem cell transplantation, allogeneic limbal stem cell transplantation[28-31]. Now, stem cell transplantation is the most optimal method in severe cases and in total LSCD to restore a healthy corneal surface. Amniotic membrane transplantation, coupled with conjunctival epitheliectomy, could be a therapy in partial LSCD[32]. At present, LESCs transplantation is the main treatment method, but the lack of LESCs donor has limited the application of LESCs transplantation[33]. Though somatic stem cells have been applied to recover the ocular surface, the long-term clinical results have indicated that it is not be overly encouraging[34-36].

EMBRYONIC STEM CELL DERIVED CORNEA EPITHELIAL-LIKE CELLS

Human pluripotent stem cells (hPSCs) could be an alternative application in many fields. They have a wider differentiation potential and limitless self-renew than tissue-specific stem

cells and they providing an unlimited source of cells. Because the capability of pluripotent ESCs to generate multiple cell types and their unlimites expansion potential, the application of them for tissue engineering may provide advantages over traditional sources of progenitor cells. Recently the development of stem cell technology provides a new method for LESC transplantation[37]. There were a couple of approaches for ESCs to differentiate into corneal epithelial cells, such as Pax6 gene transfection, microenvironment simulation and induction factor interpolation[17,38]. Homma *et al*[39] first reported epithelial progenitors were successfully induced in 8d by culturing mouse ESCs on type IV collagen and the reconstruction of mice corneal surface is also feasible. These progenitors expressed corneal epithelial cells specific gene, keratin (K) 12. More importantly, complete re-epithelialization of the corneal surface occurred within 24h after transplantation into damaged cornea[39]. Later, mouse ESCs were found to be capable of differentiating into a monolayer of epithelium-like cells. These corneal epithelium-like cells were induced by Pax6 gene expression of mouse ESCs[38]. Ahmad *et al*[40] first reported differentiation of human ESCs into corneal epithelial-like cells by *in vitro* replication of the corneal epithelial stem cell niche. Since then, several other studies[41-43] have been published, all relying on different types of undefined or animal-derived components, such as amniotic membrane, feeder cells, or conditioned medium, alone or in combinations. Most recently, a serum free- and Xeno-free protocol has been reported to initiate differentiation towards LESC-like cells with induction medium and hPSC medium modified by decreasing KO-SR concentration, increasing bFGF concentration, and adding transforming growth factor β (TGF-β) inhibitor SB-505124, Wnt inhibitor IWP-2. After the induction stage, cell aggregates were plated onto plates coated with human placental collagen IV, and maintained in a defined and serum-free corneal epithelium medium CnT-30. After a total of 30-35d, the proteomics of human PSCs-derived LESCs are similar to native corneal epithelial cells[44]. However, potential immune rejection and ethical issues have limited the application of ESCs. What's more, more researches about MSCs arrests our attention. MSCs have been found to differentiate into corneal epithelioid cells when they were co-cultured with corneal stromal cells and transplanted into ocular surface[45]. MSC could also inhibit postoperative corneal inflammation and angiogenesis. However, the reduction of differentiation ability after multiple generations limits their clinical application[37,46-47].

INDUCED PLURIPOTENT STEM CELLS

As previously mentioned, in 2006, Takahashi and Yamanaka[3] developed iPSCs through reprogramming several types of somatic cells by introducing four transcription factors (Oct3/4, Sox2, c-Myc, and Klf4) into these somatic cells under ESC culture conditions. For the first time, they demonstrated induction of pluripotent stem cells from mouse embryonic or adult fibroblasts by reprogramming. Since then, more academic researches are conducted profoundly. The finding allows the creation of pluripotent cells from homograft somatic cells of patient straightforward. With the development of iPSCs research technology in the last decade, a great progress have been made in the following aspects, such as cell source diversity used for reprogramming, function of transcription factors during the reprogramming, safety of vectors used for the delivery of transcription factors, advances in differentiation efficiency. iPSCs can provide new approaches for autologous cell-based therapy, possibly organ replacement treatments and iPSCs generated from patients offer profound insight into the mechanisms of disease. The study will impact human disease modeling, drug discovery and testing[48]. Studies have confirmed that iPSCs can differentiate into multiple cell types: islet cells, cardiomyocytes, nerve cells, retinal cells, and so on, which offered new therapy strategy for the patients of diabetes, myocardial infarction, Parkinson's disease, and retinal disease. Besides that it represents a promising resource for new and ongoing studies of ocular morphogenesis.

INDUCED PLURIPOTENT STEM CELLS-DERIVED CORNEAL EPITHELIAL CELLS

Over the past few years, the research on iPSCs used as a source of regenerative medicine developed rapidly. Over the past decade, progress in stem cell researches for blinding diseases is now being applied to patients with retinal degenerative diseases. iPSCs technology has opened a new avenue for treating various diseases by using patient specific cells, eliminating the risk of immune rejection after transplantation of the patient's autologous iPSCs-derived cells, such as retinal pigment epithelial (RPE) cells[49]. The use of iPSCs-derived corneal epithelial cell may provide an unlimited source of cells and circumvent the issues, which exist in human ESCs-derived corneal epithelial cell. Recently, by mimicking the environmental niche of limbal stem cells, several protocols have been developed, aimed at the differentiation of iPSCs into the corneal epithelial lineage[50-51]. More recently, small molecule driven protocols have become available resulting in generation of corneal epithelial-like cells within 6wk[15].

Great achievements have been made in deriving iPSCs into corneal epithelial cells, especially remarkable researches of differentiation methods. In 2012, for the first time Hayashi *et al*[42] demonstrated a strategy for the differentiation of human iPSCs into corneal epithelial cells, and also noted that the epigenetic status was associated with the propensity of differentiation of iPSCs into corneal epithelial cells. In their study, they used stromal cell-derived inducing activity (SDIA) differentiation method[52-53] to induce corneal epithelial cells from the iPSCs, as Pax6+/K12+ corneal epithelial colonies

were observed after prolonged differentiation culture (12wk or later) in both the human adult corneal limbal epithelial cells (HLEC)-derived iPSCs (L1B41) and human adult dermal fibroblast (HDF)-derived iPSCs (253G1). The corneal epithelial differentiation efficiency was higher in L1B41 than that in 253G1. There was no significant difference between L1B41 and 253G1 iPSCs was noted in methylation status of the corneal epithelium-related genes, such as K12, K3, and Pax6[42]. However another study found that when the gene methylation patterns of iPSCs in comparison to their parental cells, limbal-derived iPSCs had fewer unique methylation changes than fibroblast-derived iPSCs. Limbus-derived iPSCs cultured for 2wk on human amniotic membrane (HAM) developed markedly higher expression of putative LESC markers keratins 14, 15, and 17, ABCG2, ΔNp63α, N-cadherin, and TrkA than did fibroblast iPSCs[54]. The study also described a different approach, differentiating LESC-derived iPSCs without feeder cells but using biological supports that were identical (denuded organ-cultured corneas) or similar (denuded HAM) to their natural niche. They emphasized the importance of niche factors, including underlying BM and stromal cells[54-55]. Then in 2013, Yu *et al*[56] co-cultured iPSCs in the presence of additional factors bFGF, EGF and NGF, activated keratin K12 expression (a marker of corneal epithelial cells) and downregulated Nanog with corneal limbal stroma, which is separated by a transwell membrane. They found that after 12d of differentiation procedure, by replication of a corneal epithelial stem cell niche, mouse iPSCs could differentiate into corneal epithelial-like cells. Through scanning electron microscopy they found differentiated iPSCs also had multiple microcilia, like normal mouse corneal epithelial cells. Differentiated iPSCs were smaller than cultured epithelial cells was the difference between the two types of culture. Besides that they found iPSCs-derived epithelial cells expressed K12, a specific marker of corneal epithelial cells, and pax6, which was necessary for early development of eyes. The expression of P63, a marker of corneal epithelial stem cells, was activated after co-culture. The expression of pluripotent gene, Nanog, decreased after differentiation[56]. These studies will help successor to explore more efficient, especial and handy methods for differentiation from iPSCs to corneal epithelial cells.

However, the above studies have a tissue-specific focus solely and fail to reflect the complicacy of whole eye development. In the past, we also have made progress at whole eye level. Hayashi *et al*[57] demonstrated the generation of ocular cells in a self-formed ectodermal autonomous multi-zone (SEAM) from iPSCs. In a way, the concentric SEAM mimicked whole-eye development. The approach also demonstrated the cells in the SEAM could be purified by fluorescence-activated cell sorting (FACS) and expanded *in vitro* to form a corneal epithelium that recovered function in an experimentally induced animal model of corneal blindness. In their protocol, they adopted a 2D culture system with the laminin 511 E8 fragment as a substrate for ocular cell growth, and they used a serum-free differentiation medium to promote autogenic eye-like differentiation of human iPSCs[57]. The new protocol for differentiation of iPSCs to corneal included human iPSCs autonomous differentiation, purification, and subsequent differentiation. Especially the culture techniques did not need feeder cells or fetal bovine serum. Besides, the protocol used a combination of antibodies to purificate iPSCs-derived corneal epithelial stem and progenitor cells by FACS-sorting. So that the final product was devoid of non-corneal iPSCs-derived epithelial cells, and of cellular impurities that might originate from feeder cells[58]. Recently a direct transdifferentiation approach was established to circumvent the intermediate state of pluripotency (iPS-stage). The resulting cells, which are obtained directly by transdifferentiation from fibroblasts to limbal cells, displaied corneal epithelial cell morphology and corneal epithelial markers experssion. They showed a direct transdifferentiation of human dermal fibroblasts into the corneal epithelial lineage that may serve as a source for corneal epithelial cells for transplantation approaches and avoided tumorigenic potential[50].

INDUCED PLURIPOTENT STEM CELLS-DERIVED CORNEAL CELLS FOR CORNEAL DISEASE MODELING

Above studies include how to harvest stem cells, the source of stem cells, their progeny, and the differentiation method of iPSCs. The following studies enhanced clinical success by discussing iPSCs-derived keratocytes to promote comprehending cornea disease mechanisms, establishing corneal disease organoid models or keratoconus (KC) model. iPSCs-derived keratocytes allow for novel approaches in disease modeling and drug development platforms. Keratocytes derived from iPSCs of patients offer an autologous source, which will be used as a research tool for understanding cornea disease mechanisms. One study indicated this iPSCs-model system allowed for the identifying of miR-450b-5p as a molecular switch of Pax6, a major regulator of eye development. MiR-450b-5p and Pax6 were found reciprocally distributed at the presumptive epidermis and ocular surface, respectively. It suggests that by repressing Pax6, miR-450b-5p triggers epidermal specification of the ectoderm, while the absence of miR-450b-5p accompanied with Pax6 expression allows ocular epithelial development[51]. A recent study showed human iPSCs-derived organoids through sequential rounds of differentiation shared features of the developing cornea, appeared translucent with a clear or a dense center, and harbored three distinct types of cells with expression of key epithelial, endothelial and stromal cell markers. Cornea

organoid cultures could be a powerful 3D model system for investigating the developmental process of corneal and their disruptions in pathological conditions[59]. Another study made a model of KC using iPSCs generated from fibroblasts of both normal human corneal stroma and KC with a viral vector. The author found that the inhibition FGFR2-Pi3-kinase pathway affected the AKT phosphorylation, and subsequently affecting the keratocytes survival signals, while the normal group did not affect the keratocytes survival signals. A potential mechanism for the KC-specific decreased cell survival and apoptosis of keratocytes could be the inhibition of the survival signals[60]. Therefore, continuous development in this area will lead to a novel understanding of the multifactorial and complex corneal disease.

FUTURE PERSPECTIVE

Stem cell technology is a promising approach to provide a limitless source for cell replacement therapy of LSCD. Increasing number of studies reported differentiation of iPSCs into corneal epithelial cells and successfully established reproducible protocols. Transplantation of the iPSCs-derived corneal epithelial cell could recover function in an animal model of corneal blindness[57]. However, the current method of differentiation is time-consuming, expensive, and inefficient, which has not yet reached the standard and level of clinical application. So the protocol of iPSCs differentiation into corneal epithelium should be improved continually. At present, iPSCs-derived corneal epithelial cell is only demonstrated to repair the corneal function in animal model. No clinical trial has been reported to confirm its efficacy and safety of cells post-transplantation in human. As we known, the undifferentiated iPSCs have limitless proliferation potentials and/or tumorigenic transformed cells, the tumorigenic potential of iPSCs-derived corneal epithelial cell has not yet been tested, which is attributable to contamination by undifferentiated iPSCs[61]. The risk of teratoma formation associated with the use of iPSCs hinders applications from lab into clinics. All of these prevent iPSCs-derived corneal epithelial cells from the clinical treatment of patients with corneal disease. Although in the past five years, the technology of iPSCs differentiation into corneal epithelial cells has made great progress, but there are some important obstacles that must be overcome. It still has a long way to go before iPSCs-derived corneal epithelial cells can be used in clinic.

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