

Current focus of stem cell application in retinal repair

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research. Embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and adipose derived mesenchymal stem cells (ADMSCs) are the focus in current endeavors as a source of different retinal cells, such as photoreceptors and retinal pigment epithelial cells. The aim is to apply them for cell replacement as an option for treating retinal diseases which so far are untreatable in their advanced stage. ESCs, despite the great potential for differentiation, have the dangerous risk of teratoma formation as well as ethical issues, which must be resolved before starting a clinical trial. iPSCs, like ESCs, are able to differentiate in to several types of retinal cells. However, the process to get them for personalized cell therapy has a high cost in terms of time and money. Researchers are working to resolve this since iPSCs seem to be a realistic option for treating retinal diseases. ADMSCs have the advantage that the procedures to obtain them are easier. Despite advancements in stem cell application, there are still several challenges that need to be overcome before transferring the research results to clinical application. This paper reviews recent research achievements of the applications of these three types of stem cells as well as clinical trials currently based on them.

Key words: Retina; Adipose derived mesenchymal stem cells; Embryonic stem cells; Induced pluripotent stem cells; Cell therapy

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Core tip: Several retinal diseases are based on different retinal cell layer degenerations. Some of them have no treatment to date. Advances in stem cell therapy are considered a realistic option. In contrast to the early stage, the late stage of these diseases requires treatment techniques based on cell replacement. Currently, a few are in clinical trials using the sources of embryonic stem cells, induced pluripotent stem cells and adipose derived mesenchymal stem cells. The focus is mainly to enhance the output of these techniques, securing faster implementation and patient safety. Nevertheless, there

Abstract

The relevance of retinal diseases, both in society's economy and in the quality of people's life who suffer with them, has made stem cell therapy an interesting topic for

are many challenges still to resolve.

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INTRODUCTION

The retina is a very complex peculiar tissue which has ten different layers, namely retinal pigment epithelium (RPE), photoreceptor outer segments, outer limiting membrane, outer nuclear layer, outer plexiform layer, inner nuclear layer, inner plexiform layer, ganglion cell layer, nerve fiber layer and inner limiting membrane. The cells and membranes of these layers are interconnected and in constant physiochemical communication to maintain retinal homeostasis, anatomy and functional integrity. An alteration or damage in any of these layers can impair these communications, thereby affecting the normal homeostasis, anatomy and functions of the retina^[1]. It can result in an onset of retinal diseases which can lead into visual impairment or blindness.

An example of this is the dry form of age-related macular degeneration (AMD) in which the damage affects four interconnected layers: the RPE, photoreceptor, Bruch's membrane and the choriocapillaris^[2]. Post stimulation of damage events, the RPE degenerates finally and consequently the photoreceptor also degenerates, leading to blindness^[2]. In addition to AMD^[2-4], currently there are many other retinal diseases, such as retinitis pigmentosa^[4,5] and diabetic retinopathy^[4,6], and eye diseases, such as glaucoma and non-arteritic anterior ischemic optic neuropathy, in which damage occurs on different retinal layers (Table 1). Thereby, it provokes retinal cell degeneration that results in vision loss in patients with the disease. Remarkably, some retinal diseases have no treatment yet although their impacts on daily life and social and economic consequences are very high.

All the retinal diseases mentioned already cause degeneration and loss of the retinal cells. It is logical to think that the replacement of damaged retinal cells with healthy ones could be a therapeutic option but it is necessary to have a suitable source of the cells. Stem cells are very prominent and promising for treatment development because they have shown very positive results so far. They have the advantage over the patient's adult retinal cells, solving the various issues of treatment development, including the gene related issues. The impact of retinal diseases on daily life and the social and financial consequences enforce the need for research of stem cells-derived therapies applied to human trials as fast as possible without forgetting issues of the safety and ethics^[7]. Because of this, there are several ongoing research studies about this topic.

This review focuses on recent achievements of the applications of embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and adipose derived mesenchymal stem cells (ADMSCs) in retinal repair. It also describes the clinical trials based on the use of these three types of stem cells and deals with the disadvantages of the use of each of these stem cells.

Stem cells have also shown neuroprotective effects on degenerating retinal cells which could be applied to delay or stop the cellular damage events; thus, it could be useful to treat retinal diseases in their early stage. However, this review mainly focuses on cell replacement therapy which is applicable in the late stage of retinal degeneration diseases. This review article is based on the approaches used in published articles. It does not describe the biological mechanisms involved in each approach in detail but only describes recent advances in research that seem interesting for performing an *in vivo* test, thus transferring the results to the clinic in a short period.

ESCs

ESCs are pluripotent stem cells which means they can differentiate into ectoderm, mesoderm and endoderm cells. However, some ESC cells lines show more differential potential towards a particular lineage than others do. For example, it has been found that human ESC H7 has good differentiation potential into beating cardiomyocytes but is weak into ectodermal lineages^[8], whereas human ESC Regea 08/023^[9] has better ectodermal than beating cardiomyocytes differentiation potential^[8]. Which lineage of the ESC and protocol will be used in the experiments to achieve the research objective have to be considered in the decision making.

The ESC pluripotent characteristics have allowed several studies to be performed in which differentiating these cells into both neural^[10-12] and epithelial retinal cells^[13-15] has been achieved. A very crucial step is to successfully achieve establishing a method of ESC differentiation to obtain a good number of healthy differentiated retinal cells with no gene related issues because, as described above, different retinal layers are affected with executed cellular degeneration events. A specific type of cells to replace a retinal layer is needed. On the other hand, ESCs have also been used to obtain neural^[16,17] stem cells, multipotent cells which can differentiate into limited cells types including neuroretinal cells.

ESC differentiated cells could be used to replace the damaged cells of the retina. There are many published methods for this purpose. However, it is very important that the cells can be grown and differentiated in standard culture conditions. The viral-free standard culture conditions are preferable to minimize the risks associated with a virus in cellular transplantation. ESCs have been used to obtain neural stem cells by using small molecules such as CHIR99021 and SB431542. It

Table 1 Summary of retinal diseases and the type of cells affected in each case

Retinal disease	Type of cells affected
Age-related macular degeneration	Photoreceptors and retinal pigment epithelial cells
Glaucoma	Retinal ganglion cells and their axons
Non-arteritic anterior ischemic optic neuropathy	Retinal ganglion cells
Retinitis pigmentosa	Photoreceptors
Diabetic retinopathy	Neural retinal cells

has been found that they protect photoreceptors and visual function. Hence, obtaining this type of cell by using small molecules has the advantage that they are obtained efficiently and without the use of virus which could produce a risk^[4]. Thus, they could be used as a potent cell source for treating degenerative retinal diseases^[16].

ESCs have been also used to obtain progenitor retinal cells which can be differentiated to specific retinal cells such as photoreceptors. There are many published methods for this purpose but identifying an effective and efficient method is crucial if performing a human clinical trial. Amirpour *et al.*^[17] compared three methods of obtaining photoreceptors using co-culture of the RPE with retinal progenitor cells differentiated from ESCs. In two of them, the RPE were indirectly co-cultured on filters for 1 and 2 wk respectively, with retinal progenitors cells differentiated from ESCs. The filter blocked the two layers coming in to physical contact, although they were in chemical communication using the different cell secreted biomolecules. The third one was a direct co-culture of both types of cells for 2 wk. They concluded that direct co-culture with the RPE improved the expression of late photoreceptor markers such as arrestin. In another method, Decembrini *et al.*^[10] produced a transgenic mouse ESC line following a three-dimensional (3D) culture method. The combination of these transgenic cells coupled with a 3D culture system allowed achieving the production of a secure and renewable source of photoreceptors compatible with transplantation^[10]. Previously, it was demonstrated that ESC-derived photoreceptor precursor cells were able to integrate when transplanted into degenerated adult mouse retina. In addition, the transplanted cells matured toward outer segments and formed synaptic connections^[11]. In this study, the 3D neuroepithelial cyst culture system was used to achieve the differentiation of ESCs into photoreceptor precursors. In addition, current investigations showed that hypoxia seems to play an important role in making differentiation of mouse ESCs towards the photoreceptors more effective and efficient. Thus, the study of using mouse ESCs culturing under hypoxic conditions provides an important method that can be used to improve modeling of retinogenesis *in vitro*^[12]. Thus, to obtain photoreceptors and in all studies in general, the method used to culture the cells is crucial for the results obtained. Comparing the results of different previous studies for planning new strategies of a method development should be considered.

These studies and their produced results are essential for advances in photoreceptor replacement therapy. As stated above, ESCs are a source of neural stem cells^[16,17] and photoreceptors^[10-12] but they are also able to differentiate into RPE cells^[13-15] which play an important role in the pathogenesis of many retinal diseases, including AMD. Diniz *et al.*^[13] studied the survival of human ESC-derived RPE transplanted in the subretinal space of immunocompromised nude rats. The results of this study^[13] showed that these cells could survive for several months and the obtained results were enhanced when cells were transplanted as a polarized monolayer of the hESC-RPE rather than as a cell suspension. Nevertheless, RPE is a layer of polarized cells which execute different functions at the apical and basal portion.

Most standard culture conditions of stem cell differentiation methods contain animal generated products such bovine serum, fetal calf serum, *etc.*, as nutrient supplements. However, when the cells are transplanted in humans, animal serum could result in host rejection^[18-20]. Therefore, serum-free medium was used in many studies. During the growing and differentiation of ESCs into RPE under serum-free conditions, the cells were found to express several genes which encode a family of proteins known as aquaporins. These proteins are responsible for allowing passive water transport through the RPE^[14]. Thus, this study added a property to ESC differentiated RPE which is essential for retinal homeostasis and functional integrity. Cell replacement therapy is very effective when it repairs the damaged functional properties of a cell layer as well as acquiring the cell phenotype. Hence, this is an important area of study in which the search for effective methods are currently focused^[3,15].

Recently it was reported, as stated above, that it is possible to generate RPE from ESCs using a 3D neuroepithelial cyst culture of these stem cells. This method could also be used to produce other types of neuronal cells in addition to RPE^[15]. It is also very favorable to minimize the host rejection issues if a cell culture method can produce different types of cells.

Despite all the advantages of ESCs, tumor formation is considered an important risk for the clinical application of these stem cells^[21]. Hence, teratoma formation must be studied and controlled for each method before successfully carrying out a clinical trial^[22]. It has been proved that the use of a method which achieves differentiation of ESCs and thus secures the loss of its pluripotency is related to less tumor growth when

the cells were transplanted in animals^[12,13]. The study showed a lack of tumors in immunocompromised nude rats transplanted with the well differentiated ESC-derived RPE. It supports the theory that the risk of this side effect could be minimized in immunocompetent recipients^[13]. It found that WNT signaling cascade plays an important role in tumor formation and therapeutic functionality of ESC-derived retinal progenitor cells^[21]. Such studies exploring signaling cascade roles could support developing an effective cell replacement therapy. Nevertheless, ethical concerns, limited cell sources and the related high incidence of malignant transformation restrict the wide application of ESCs in retinal repair.

Currently, there are some registered clinical trials using ESCs to treat retinal diseases (<http://www.clinicaltrials.gov/>): (1) (NCT01469832) Safety and Tolerability of Sub-retinal Transplantation of Human Embryonic Stem Cell Derived Retinal Pigmented Epithelial (hESC-RPE) Cells in Patients with Stargardt's Macular Dystrophy (SMD); (2) (NCT01691261) A Study of Implantation of Human Embryonic Stem Cell Derived Retinal Pigment Epithelium in Subjects with Acute Wet Age Related Macular Degeneration and Recent Rapid Vision Decline; (3) (NCT01674829) A Phase I / II a, Open-Label, Single-Center, Prospective Study to Determine the Safety and Tolerability of Sub-retinal Transplantation of Human Embryonic Stem Cell Derived Retinal Pigmented Epithelial (MA09-hRPE) Cells in Patients With Advanced Dry Age-related Macular Degeneration (AMD); (4) (NCT02122159) Research With Retinal Cells Derived From Stem Cells for Myopic Macular Degeneration; (5) (NCT01344993) Safety and Tolerability of Sub-retinal Transplantation of hESC Derived RPE (MA09-hRPE) Cells in Patients with Advanced Dry Age Related Macular Degeneration (Dry AMD); (6) (NCT01345006) Sub-retinal Transplantation of hESC Derived RPE (MA09-hRPE) Cells in Patients with Stargardt's Macular Dystrophy; and (7) (NCT01625559) Safety and Tolerability of MA09-hRPE Cells in Patients with SMD.

INDUCED PLURIPOTENT STEM CELLS

Human induced pluripotent stem cells (hiPSCs), generated by somatic cells reprogramming, have been a strong attractive focus of researchers in recent years. This is due to its feasible application in transplantation for cell replacement therapies for retinal diseases. However, it needs intensive investigation in the laboratory and subsequently in early-phase human clinical trial to improve the differentiation, transplantation and integration of the cells, with the goal of obtaining maximal therapeutic efficacy^[7].

Like ESCs, iPSCs are able to differentiate in several retinal cells, such as the photoreceptors^[23], RPE^[24-29], retinal ganglion like cells^[30,31] and retinal progenitor cells^[27,32]. In addition, these stem cells can form optic vesicle-like structures that could be used as a model

system to study the development of the retina^[33].

Using iPSCs of swine, their differentiation into photoreceptors in cell culture has been achieved. In addition, when these cells were transplanted into the subretinal space of pigs, it was found that they integrated in the retina previously damaged with iodoacetic acid^[23]. On the other hand, this study used the pig as an animal model for a retinal stem cell transplantation study, which could be taken into consideration for future transplantation studies. The pig and human eyes show some similarities in anatomical and histological structure and thus provide the opportunity to translate the research results to human clinical use in a short period.

Regarding iPSC derived RPE, the sheets of the differentiated RPE cells have been found to be very similar in morphology and physiology to the native RPE, which is not the case for the RPE cells in suspension. Furthermore, they have other advantages, such as traceability due to the autologous property, the strong coverage of the lesion when they are transplanted and reduction of the risk of graft rejection, mainly in diseases with a disrupted blood-retinal barrier^[24]. Despite the mentioned advantages, the surgical method of sheet transplantation is more complicated and invasive in comparison with cell suspension transplantation^[24]. This important issue must be resolved before planning a clinical trial with any constructed cell sheet.

The RPE plays an important role in the retina, regulating many functions including its participation in the visual cycle. Thus, it is a crucial layer for maintaining vision function. Human iPSC derived RPE in cell culture express proteins related to visual cycle. In blind mice, these RPE cells with a functional visual cycle were transplanted and it was found that they reestablished in part the visual function of mice. This result supports the possibility of the use of these cells for treatment of retinal degenerative diseases^[25]. Generating a functional monolayer of RPE cells showing native RPE characteristics, such as pigmentation, morphology, specific marker expression, polarized membrane, vascular endothelial growth factor secretion and phagocytic activity, in large amounts from human iPSCs by using simple methods has been achieved. The modifications done in this simple method with respect to the standard method were the amplification of human iPSC by clonal propagation. Furthermore, enrichment of the RPE cells has been achieved by serial passages as an alternative to mechanical picking^[26]. In another study, developing an easy method to differentiate iPSCs into the RPE and also into retinal progenitor cells has been achieved. Thus, it provides useful data for reducing the time necessary for translating the results to the clinic. This method consists of culture of confluent human iPSCs without using Matrigel or serum in a proneural medium^[27]. However, it is necessary to control cell passaging during their culture for maintaining the features of human iPSC derived RPE. It was found that in fourth passage of culture the morphological and functional characteristics of the cells were changed^[29].

Thus, these studies provide enough advances for exploring the feasible application of human iPSC derived RPE.

Like ESCs, iPSCs were also used to obtain neural progenitors. In both *in vitro* and *in vivo* studies, neural progenitors derived from human iPSCs showed their differentiation potential into retinal cells. In a study^[32], these cells were transplanted into rats with injured optic nerves and the results showed that there was a rescue of injured optic nerves due to the stem cell-mediated neuroprotective effect on the retinal ganglion cells. This confirmed that iPSCs maintain a stem cell neuroprotective property that could be applicable in developing neuroprotective property based treatments for retinal diseases. However, as mentioned earlier, this review focuses only on cell replacement therapy for retinal diseases.

In addition to these three types of cells, it is also possible to produce retinal ganglion like cells from iPSCs. Applying methods for induction of the adenoviral genes, retinal ganglion like cells were produced faster than what could be produced using lentiviruses. This was due to a more efficient reprogramming process executed inside the cell when mediated by adenoviruses. There was no viral genomic integrations if adenoviruses were used in the study, *i.e.*, these cells were free of exogenous gene integration. In this study, the resulting cells were derived from the mouse fibroblast and they showed electrophysiological characteristics^[30]. The fibroblasts were obtained more easily in sufficient amounts for a patient than the ESCs and their successful reprogramming resolves to an extent the issues related to finding an appropriate cell source and the ethics compared to ESCs. Recently it was reported that the ciliary body derived cells showed higher reprogramming efficiency than the fibroblasts for producing iPSCs; thus, it could be also overcome the issues related to the ocular origin^[34]. However, the concerns related to the autologous cell nature and the surgical complications of obtaining the cells from a patient could discourage the use of ciliary body derived cells.

Furthermore, it has been hypothesized that iPSCs play a role in retinal ischemia and reperfusion (I/R) injury. In a rat model of this retinal disease, iPSCs without c-Myc (oncogene that contributes significantly in tumor formation) were injected subretinally^[35]. Results of this study showed that transplanted non-c-MYC iPSCs rescued I/R retinal damage induced in rats and reduced the tumorigenicity. Thus, it confirms the suitability of iPSCs for treating this disease. Besides this disease, transplantation studies in animals are continuously showing positive results for the feasibility of iPSC application in cell replacement therapy for retinal diseases.

iPSCs are an important renewable source of cells, with all that it means for both medicine and basic research^[36]. Despite numerous challenges that remain, iPSCs could be a realistic option for cell replacement treatment in the future for patients with retinal diseases

that are still untreatable and need replacement of the damaged cells. Thus, it could play a significant role in research strategies to slow down or stop the disease progression or could completely treat the disease and probably enhance visual function^[37].

As stated above, there are still challenges for the application of iPSCs^[37] which need to be overcome before translating the advances to the clinic, such as the risk of leading to malignant tumors^[36]. Unlike ESCs, iPSCs have the advantage of being used for autologous transplantation. Consequently there will be fewer immune rejections, although on the other hand, personalized cell therapy has a high cost in terms of both economics and time^[38]. Therefore, several researchers have a common goal^[26,27] to develop an easy method that could reduce these disadvantages.

Currently there are some registered clinical trials using iPSCs to treat retinal diseases (<http://www.clinicaltrials.gov/>): (1) (NCT02162953) Development of Induced Pluripotent Stem Cells from Patients with Best Disease and Other Inherited Retinal Degenerative Diseases; and (2) (NCT01432847) Generation of induced pluripotent stem (iPS) Cell Lines from Somatic Cells of Participants with Eye Diseases and from Somatic Cells of Matched Controls.

ADMSCs

ADMSCs are a source of adult stem cells which can be obtained by a minimally invasive procedure, liposuction, followed by adequate processing to achieve a population of cells with the desired characteristics^[4]. This is an easier, more accessible and safer source of mesenchymal stem cells than the bone marrow^[6]. In addition, they can be easily expanded under standard culture conditions without progressive culture and enrichment and with a lower risk of contamination^[6,39]. Thus, this is a very important source for obtaining mesenchymal stem cells (MSCs) in large numbers. MSCs are highly significant for patients with retinal diseases, including diabetic retinopathy (DR), AMD and retinitis pigmentosa (RP)^[4].

DR is a common ocular complication in diabetic patients which involves microvascular and neurodegenerative diseases^[6]. ADMSCs could be an effective part of the therapy for the DR since it has been found that these cells homed in to the injured retina and differentiated to form astrocytes and photoreceptor-like cells when injected intravenously in streptozotocin diabetic rats. Besides, ADMSCs were able to reduce blood glucose levels and repair the blood-retina barrier breakdown that occurs in this disease^[6].

AMD is a neurodegenerative disease associated with aging which progressively leads to loss of the central vision. Damage is produced at the RPE, from the posterior extended to the photoreceptor. The RP involve a set of hereditary diseases characterized by degeneration of both types of photoreceptor cells, rods and cones. Patients with RP progressively lose vision, night vision in adolescence, side vision in young adulthood and central vision in later

Table 2 Summary of the routes of administration of stem cells

Type of stem cells	Route of administration	Species used	Ref.
ESC-neural stem cell	Subretinal and intravitreal	Rat	Lu <i>et al</i> ^[16]
ESC- retinal progenitor cells	Subretinal space	Mouse	Cui <i>et al</i> ^[21]
ESC- photoreceptors	Subretinal space	Mouse	Decembrini <i>et al</i> ^[10] Gonzalez-Cordero <i>et al</i> ^[11]
ESC-RPE	Subretinal space	Rat	Diniz <i>et al</i> ^[13]
iPSC	Subretinal space	Rat	Fang <i>et al</i> ^[35]
hiPSC-RPE	Subretinal space	Rat	Kamao <i>et al</i> ^[24]
		Mouse	Maeda <i>et al</i> ^[25]
iPSC-photoreceptors	Subretinal space	Pig	Zhou <i>et al</i> ^[23]
hiPSC-neural progenitors	Intravitreal	Rat	Satarian <i>et al</i> ^[32]
ADMSC	Intravitreal	Rat	Haddad-Mashadrizeh <i>et al</i> ^[43]
ADMSC	Intravenous	Rat	Yang <i>et al</i> ^[6]

ESC: Embryonic stem cells; iPSC: Induced pluripotent stem cells; ADMSC: Adipose derived mesenchymal stem cells.

life^[5]. Both diseases involve photoreceptor cell death. Therefore, studies of MSCs mainly target two aims: cell replacement and neuroprotection. These stem cells have been differentiated into many cell types, including retinal progenitor cells^[40] and RPE^[41], which confirms their application in cell replacement therapy for retinal diseases. In addition, these cells have been found to exert paracrine effects showing neuroprotective effects on RPE damage *in vitro*^[42]. These properties of these cells were confirmed in animal transplantation studies. Hence, ADMSCs are a potent source of cells with properties that could be applicable in developing treatment.

In a transplantation study, when human ADMSCs were injected in the vitreous cavity, they survived and integrated in the rat eye but were found to cross the blood-retina barrier and migrate to non-targeted organs. Thus, it could be a risk for transplantation in patients. This must be taken into consideration in the strategies of further investigations and before performing a clinical trial^[43]. These MSCs show many advantages in terms of a good source of cells with minimum complexity to obtain in sufficient amounts and develop treatments based on the application of their properties. Nevertheless, there are still several questions about these cells that need to be answered. Therefore, further research is needed to understand the action mechanisms of these cells and when they could be transplanted^[39].

Currently there are some registered clinical trials using ADMSC to treat retinal diseases (<http://www.clinicaltrials.gov/>): (1) (NCT02024269) Study to Assess the Safety and Effects of Cells Injected Intravitreal in Dry Macular Degeneration; and (2) (NCT02144103) Effectiveness and Safety of Adipose-Derived Regenerative Cells for Treatment of Glaucomatous Neurodegeneration.

ROUTES OF ADMINISTRATION OF ESCs, iPSCs AND ADMSCs

The present review describes both *in vitro* and *in vivo* studies. In the latter, researchers have used different routes of administration of the stem cells for evaluating

the safety and efficacy of their approaches. The subretinal space^[10,11,13,16,21,23-25,35] is a place most frequently used to inject the stem cells, but intravitreal^[16,32,43] and intravenous^[6] administration have also been used. Table 2 presents a summary of the routes of administration of each type of stem cells discussed in this review.

CONCLUSION

In the last few years, there have been important advances in the area of stem cells which have made their application in cell replacement feasible. It could be used as a cell replacement therapy for untreatable retinal diseases where cells cannot be recovered due to cell damage. A type of stem cells, the MSCs, are useful as neuroprotective agents, but also, the use of stem cells would not prevent apoptotic progression (the main cause of these diseases) and this is an important and still unresolved problem. The advances in both approaches should be taken into consideration as realistic therapeutic options for developing treatments for untreatable retinal diseases. However, before applying these advances to clinical therapy, it is necessary to perform extensive investigations to improve the processes of obtaining, differentiation and implantation of these cells to overcome the challenges which are still present.

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