Review paper

Embryonic stem cells and retinal regeneration

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Abstract

The potential of embryonic stem cells (ESCs) to differentiate into various cell types make them prime candidates to play an important role in the future of regenerative medicine. A wide range of retinal degenerative diseases which are the leading cause of irreversible blindness worldwide may be potentially treated by stem cell therapies. Common retinal neurodegenerative pathologies mainly affect one of three ocular cell types: ganglion cells (e.g. glaucoma), photoreceptors (e.g. retinitis pigmentosa) and retinal pigment epithelium (RPE) cells (e.g. age-related macular degeneration). This article summarizes the latest advances made in embryonic stem cell-based medicine for retinal diseases and presents some obstacles which need to be overcome before ESCs can be used safely and effectively.

Key words: *embryonic stem cells, retina, RPE, photoreceptors, ganglion cells, regenerative medicine.*

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Introduction

Embryonic stem cells (ESCs) are derived from the blastocyst inner cell mass and can differentiate into over 200 cell types from all three germ layers. Their pluripotency has been demonstrated with both mouse and human ESCs, first successfully isolated in 1981 and 1998, respectively [1]. Cultures of ESCs are characterized by the specific morphology and the presence of cell surface markers such as SSEA-1 and Pecam1, or the expression of the key transcription factors such as Oct4, Sox2, Nanog, and a several of ES cell-specific transcripts (ECATs) [1]. Astonishingly, a recent study by Canham *et al.* demonstrated that a subpopulation of ES cells can also meet a criterium of totipotency by the contribution to the extra-embryonic lineages [2].

The mammalian retina is a complex neural structure which processes visual signals. It is organized into three different nuclear cell layers that include seven major cell types with more than 50 subtypes [3]. The outer layer is populated by photoreceptor cells, the middle layer harbours bipolar, amacrine and horizontal cells and the innermost layer predominantly contains ganglion cells and displaced amacrine cells. The retinogenesis follows a chronological sequence, in which retinal ganglion cells, cone photoreceptors, horizontal cells and a population of amacrine cells are generated during early stage of retinal histogenesis, whereas bipolar cells, Müller glia and most rod photoreceptors are generated during late stages of histogenesis [4]. Despite significant limitations to clinical applications, especially due to ethical consideration and potential tumorigenicity, ESCs seem to be promising source of cells for retina replacement. Their potential for infinite proliferation makes them superior to adult stem cells in terms of production of a sufficient number of required cells.

Generation of photoreceptors from embryonic stem cells

Currently, there is no effective treatment for various ocular diseases, incuding retinitis pigmentosa, cone dystrophy and age-related macular degeneration which are characterized by photoreceptors loss leading to blindness. ES-derived photoreceptors would potentially serve as a cell replacement, neuroprotection or provide aid in better understanding of degenerative processes.

In 2002, Zhao *et al.* demonstrated that mouse ES cell-derived neural progenitors express regulatory genes that have been shown to play a role in photoreceptor differentiation, i.e. Rx, Crx, Nrl, and NeuroD and that in response to epigenetic cues a subset of them differentiate along photoreceptor lineage [5]. During the differentiation, they produce photoreceptor-specific proteins i.e.: rhodopsin kinase, arrestin, two phototransduction proteins; peripherin-

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a structural protein and interphotoreceptor retinalbinding protein (IRBP) – a protein used for retinoid transport. Thus it suggests that ES cell-derived neural progenitors recruit mechanisms normally used for photoreceptor differentiation *in vivo*. However, cells expressing photoreceptor-specific markers did not display typical photoreceptor morphology. The same group of authors described the protocol to promote differentiation of the mosue ES cell-derived neural progenitors into retinal cells, specifically the rod photoreceptors [6]. As 95% of the photoreceptor in human retina are of the rod type and they are predominantly lost in retinitis pigmentosa, their *in vitro* generation is particularly valuable [6].

The efficient induction of rhodopsin+ photoreceptor-like cells from mouse embryonic stem (ES) cells by co-culturing with chick embryonic retina tissue has also been reported. These cells expressed the transcription factor crs and a distinct increase of rod photoreceptor-specific markers, IRBP and recoverin confirmed by RT-PCR analysis [7].

In the next study, mouse ES cells were induced to a neural lineage and transplanted into the vitreous of mnd mouse eyes. Mnd mice with a defect in gene CLN8 are animal models for human autosomal recessively inherited lysosomal storage diseases – neuronal ceroid lipofuscinoses (NCLs) leading to retinal and CNS degeneration [8]. Significant incorporation of cells after intravitreal transplantation appears to depend on very specific conditions [3, 9, 10]. It apparently occurs when the development of the retina is not fully completed or when the host retina is damaged due to injury or diseases. Furthermore, in normal eyes, the inner-limiting membrane is a barrier against cells incorporation. Incorporated donor cells exhibited morphologies similar to several retinal-specific cell types including ganglion cells, amacrine cells, bipolar cells and horizontal cells. Interestingly, no donor cells acquired the morphological features of normal photoreceptors, however many of them expressed the phororeceptor-specific marker rhodopsin. Finally, although ES-derived neural progenitors were demonstrated to enhance survival of host photoreceptors, the specific mechanisms underlying this process was not apparent.

Ikeda *et al.* indicated that Rx+/Pax6+ neural retinal precursors are generated at a significant frequency from mouse ES cells in the serum-free suspension conditions (SFEB culture) combined with Wnt and Nodal antagonists (Dkk1, LeftyA), FCS, and activin treatment [11]. The induced Rx+ cells coexpress Pax6 and the mitotic marker Ki67, but not Nestin. However, whether these factors act mainly in the induction of Rx+/Pax6+ progenitors or in their growth and maintenance wasn't elucidated. In addition, the ES cell-derived precursors efficiently generate cells with the photoreceptor phenotype (rhodopsin+, recoverin+) when cocultured with embryonic retinal cells capable of the selective integration and survival in the outer nuclear layer of the retina.

Also, the *in vitro* generation of putative rod and cone photoreceptors from not only mouse but also monkey and human ES cells by stepwise treatments under defined culture conditions, in the absence of retinal tissues has been demonstrated [12].

Several laboratories have developed procedures for differentiating hESCs into retinal progenitor cells which developed further to express photoreceptor markers. In first of these studies, after intraocular transplantation of the neural precursors (NPs) derived from hESCs into rat eyes, they survived for 16 weeks, migrated large distances, and integrated with the host retina [13]. Moreover, the differentiation into cells expressing specific markers of mature photoreceptors including rhodopsin, blue cone opsin, and neural retina leucine zipper transcription factor occurred only after subretinal transplantation. This was not observed in human cells transplanted into the vitreous nor during any spontaneous *in vitro* differentiation. This observation may lead to conclusion that extrinsic cues in the subretinal microenvironment were required to support differentiation toward a photoreceptor fate.

In a next study, up to 80% of hES cells – H1 line was directed to the retinal progenitor fate and expressed a gene profile similar to progenitors derived from human fetal retina confirmed by quantitative PCR and immuno-fluorescene labeling [14]. Futhermore, cells differentiate into inner retinal neurons (ganglion and amacrine cells) with functional glutamate receptors and increased in the expression of photoreceptor-specific markers upon co-culture with retinal derived from a mouse model of Leber's congenital amaurosis (caused by a mutation in the Aipl1 gene).

In a follow-up study retinally directed hES cells have been used for transplantation into both wild type and Crx deficient mice (a model of Leber's Congenital Amaurosis) [15]. Crx is one of the earliest genes expressed by differentiating photoreceptors and its lack results in severe reduction or complete loss of components of the photo-transduction cycle, like rhodopsin, cone opsin, rod transducin, cone arrestin, and recoverin. When transplanted to newborn wild type mice, the cells integrate into all the retinal layers and express markers appropriate for the lamina in which they have settled. After transplantation into the sub-retinal space of adult mice, the cells integrate into the outer nuclear layer and express photoreceptor markers. Their morphology was very similar to the host photoreceptors, though the human cells expressed slightly lower levels of S-opsin immunoreactivity than the host mouse cones. When transplanted into the subretinal space of adult Crx –/– mice the hES cells differentiated into functional photoreceptors and restored light responsiveness. The integration into mouse retinal circuit was determined by expression of synaptophysin which is a pre-synaptic protein in the axon terminal of both rod and cone photoreceptors [16].

More recently, the successful induction of photoreceptors was achieved by co-culturing primate embryonic stem cells with ESC-derived RPE cells and the use of retinoic acid [17]. Here, for the first time ESC-derived cells have been used to induce ESC differentiation. RT-PCR analysis demonstrated the expression of retina-related gene markers such as Pax6, CRX, IRBP, rhodopsin, rhodopsin kinase, and Muschx10A. It is likely that the combined transplantation of RPE cells and ESC-derived photoreceptors may be more useful, since RPE plays an important role in maintenance of photoreceptors.

Generation of retinal pigment epithelium cells from embryonic stem cells

Retinal pigment epithelium (RPE) cells are pigment cells which form monolayers and have multiple functions, such as contributing to the blood-retina barrier, controlling nutrient and metabolite flow, re-isomerizing all-trans-retinal produced during photoconversion and phagocytosing the photoreceptor outer segments [18, 19].

In 2003, a culture system was established in which eye-like structures containing lens, neural retina, and retinal pigmented epithelium (RPE)-like cells were induced from mouse ES cells [20]. The eye-like structures were highly concentrated and partly organized. They resembled a vertical section of the vertebrate eye: a refractile cell mass containing fibrous structures was found adjacent to a cluster of pigmented epithelium-like cells. In addition, the synapse formation between neuronal cells was confirmed by showing the existence of synaptophysin concentrated in specific regions.

Furthermore, in a subsequent study an increased induction rate of the eye-like structures from mouse ES cells was achieved by the addition of Wnt2b, a putative stem cell factor in the retina [21]. In the chick embryo, Wnt2b is expressed in the lens and the ciliary marginal zone, where immature retinal precursors are present and its addition in the culture is expected to facilitate their induction. The developmental potential of RPE-like cells contained in the eye-like structures was tested after their transplantation into the chick embryonic eye. *In vivo* they developed into RPE cell monolayers expressing protein RPE65, the marker of mature RPE cells thus confirming the existence of precursors for RPE cells in the grafted eye-like structures.

In addition, primate ES cells have proved to be a source for generating cells with important RPE characteristics [22]. Gong *et al.* indicated the effects of extracellular matrix and neighboring cells on induction of hESC into RPE progenitors [23].

Evaluation of the first hESc-derived RPE cells based on trancriptomics revealed their higher similarity to primary RPE tissue than of existing human RPE cell lines D407 and ARPE-19 in terms of their gene expression profiling [24]. Moreover, they were able to phagocytosis, as well as transdifferentiation into neuronal lineage and redifferentiation into RPE-like cells.

More recently, functional analysis of phagocytosis by hESC-derived RPE cells was presented [25]. The ability of hESC-RPE to phagocytose was shown by the use of antibodies dierected against MERTK (Mer Tyrosine Kinase), which is essential for the phagocytosis of outer photoreceptors segments. In addition, co-culturing with human retina resulted in the changes in cell morphology observed under electron microscopy which typically occur when RPE cells mature. Functional and molecular characteristics of hESC-derived RPE was also described in more detail in a recent study by Bharti *et al.* [19].

The formation, expansion and expression profile of RPE-like cells derived from HESC (HESC-RPE) was studied by Vugler *et al.* [26]. They identified OTX1/2-positive cell types as potential HESC-RPE precursors and demonstrated the ability of these pigmented cells to act as their own precursors during polarised monolayers production. HESC-RPE expressed markers of both developing and mature RPE cells which included OTX1/2, Pax6, PMEL17 and RPE65 when they expanded and differentiated *in vitro*. Subsequently, they were transplanted into the Royal College of Surgeons (RCS) dystrophic rat with the primary defect of RPE cells to phagocytose the rod outer segments which is the animal model of retinal dystrophy characterized by progressive photoreceptor loss. The cells survived in the subretinal space, where they downregulated Pax6 and maintained low levels of RPE65 expression.

Several studies indicated retinal improvement, at least to some extent after transplantation of hESC-RPE into animal models of retina diseases. In the first study hESC-derived RPE was indicated to phagocytose *in vivo*, after transplantation to rats with Mertk-mutated RPE [25]. Of note, nicotinamide and Activin A were shown to promote under defined culture conditions the differentiation of hESCs into RPE. In addition, Lund *et al.* demonstrated that RPE cells derived from hESC were capable of extensive photoreceptor rescue in RCS rats with 100% improvement in visual performace over untreated controls [27]. A recent study revealed long-term (> 220 days) functional rescue measured with both visual acuity and luminance threshold response using hESC-derived RPE in both the RCS rats (animal models of age-related retinal degeneration) and Elov14 mouse (animal models of a subset of patients with Stargardt's disease caused by mutation in the Elovl4 gene) [28]. However, the mechanism whereby hESC-PRE cells rescue vision is not fully understood. ES cell-derived RPE cells were also demonstrated to restore visual function in a clinically relevant mouse model of retinitis pigmentosa [29]. Following transplantation into the rd12mouse they expressed RPE65 isomerase required for rhodopsin synthesis. Although one fourth of the mice showed increased ERG responses, RPE rescue persisted for only 4 months mostly due to the RPE graft rejection.

Importantly, it is likely that a clinically successful RPE replacement will require restoration of Bruch's membrane which is necessary for its growth [30].

Generation of ganglion cells from ESCs

Theoretically, retinal ganglion cell (RGC) replacement is especially challenging due to the need for establishment of functional synapses in the brain. Tabata *et al.* used genetic engineering to produce retinal neurons which express ganglion cell markers and electrophysiological properties [31]. It was achieved by introduction of the Rx/rax transcription factor into mouse ESCs. More recently, Kayama *et al.* obtained RGC-like cells by transfection of mouse ESCs with pax6 cDNA [32]. These cells express β III tubulin and nerurofilaments middle chain in the axon-like processes. This observation is supported by differentiation of FGF2-induced ESCs-derived neural progenitors into cells which express RGC regulators and markers such as Ath5, Brn3b, RPF-1, Thy-1 and Islet-1 [33].

Obstacles to overcome

In addition to ethical concerns and immune rejection, there is still unsolved problem of potential tumor formation by ES cells. To date, investigations into the tumorigenicity of ES-derived cells have generated variable data. A study of the NIH III immune-deficient mouse which is an immune-deficient animal model with eye pigmentation but devoid of natural killer (NK) cells and mature T and B cells showed no microscopic evidence of tumor formation after subretinal hESC-RPE transplantation [28].

No teratomas were found in study with EC-derived cells by Lamba *et al.* [15], Banin *et al.* [13], and Idelson *et al.* [18]. However, 6 weeks after the transplantation of ESCs and ESC-derived neuroprogenitors into rd12 mice eyes which mimic the slow and progressive retinal degeneration, the formation of teratomas was observed [34]. This finding is in line with the observation that neoplasia induction was present in 50% of the rhodopsin (–/–) mice which received subretinally neural precursors derived from ES cells [35].

The use of cytotoxic monoclonal antibodies directed against undifferentiated human embryonic stem cells, specifically a cell surface marker, podocalyxin-like protein-1 is one approach to solve this problem [36]. Moreover, Hara *et al.* indicated that administration of methothrexate, a folate antagonist decreased proliferative activity and tumorigenic potential of hESC-derived neurogenic cells transplanted into mouse retinas [37]. More recently, the role of the anti-apoptotic gene surviving (BIRC5) in teratoma formation by human embryonic stem cells has been demonstrated [38].However, it is still necessary to determine whether the teratogenic risk resulting from non-differentiated ES cells contaminating the graft is possible to exclude.

Another important challenge in ES cell-based therapy is the need for improvements in derivation methods to clinically relevant levels. Although, the standardization of isolation and expansion methods are still far from perfection, some progress has been made. Several studies reported on the production of photoreceptor cells under uncontaminated culture conditions [12, 14, 15]. Yue *et al.* demonstrated a culture method free of animal-derived substances which is less time-consuming and relatively less demanding [17].

Efficient methods for directing up to 80% of the human embryonic stem (hES) cells (H1 line) to the retinal progenitor fate by using a combination of noggin (a potent endogenous inhibitor of the BMP pathway), dkk1 (a secreted antagonist of the Wnt/β-catenin signaling pathway) and IGF-1 was demonstrated [14]. Interestingly, the hESC have been found to be accelerated by 3-4 weeks over the normal human embryological development. In comparison, Ikeda *et al.* have demonstrated 26% efficiency of directing mouse ES cells to RX+/Pax6+ retinal progenitors with the use of a combination of dkk1, lefty (a nodal antagonist), FCS, and activin [11]. Moreover, they have not found noggin to be effective in the mouse ES cells.

A serum-free floating culture of embryoid boy-like aggregates (SFEB) was demonstrated to be more effective in terms of inducing neural differentiation from ES cells than a stromal cell-derived inducing activity (SDIA) method [39]. Also, the culture system with the absence of animal-derived substances and feeder cells in which hESC are plated on a recombinant form of human laminin-511 was established [40]. It allows them to expand, generate a monolayer and maintain cellular homogeneity with approximately 97% OCT4-positive cells. Recently, a unique culture technique which mirrors the retina development conditions was demonstrated [41]. For the first time, three-dimensional (3D) models of retinal pigment epithelium (RPE) and early retina progenitor cells from human embryonic stem cells (hESCs) were constructed. This organized tissue structure was generated by using a cell culture insert system with an osmolarity gradient, which creates a unidirectional flow of the secreted factors from the bottom layers (RPE) towards the neural progenitor layers. In addition, the expression of several transcription factors characteristic of retinal development, such as pax6, Otx2, Chx10, retinal RAX, Brn3b, crx and nrl, as well as neuronal markers including nestin, beta-tubulin and microtubule-associated proteins were confirmed by immunohistochemistry.

So far, no single perfect marker for stem and progenitor cells is widely accepted. Therefore the use of multiple positive and negative marker combination is required. Hence, studies like a recent work by Andrew *et al.* are of special importance [42]. They present a computational method, based on algorithmic information theory which allows to predict whether retinal progenitor cells will

undergo a self-renewing or terminal division with 99% accuracy.

Despite ESCs several stem cells sources have been identified and used in approaches to regenerate retina [43]. Stem cells may play a role in cell replacement, neuroprotection but also may be a potential tool for a gene therapy and selective drug delivery to the retina. However, a number of challenges still need to be addressed before these goals become clinical reality.

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