Cell-based therapies for age-related macular degeneration: cell replacement *versus* paracrine effects

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Age-related macular degeneration (AMD) is a leading cause for severe visual loss and legal blindness in seniors worldwide. The molecular basis for the disease remains poorly understood, likely involving genetic and environment-related ocular defects. Its pathogenesis proceeds slowly, started with deposits of fatty proteins (drusen) in the Bruch's membrane, followed by gradual impairments of the posterior choriocapillaris and the anterior retinal pigment epithelium (RPE), and lead to irreversible degeneration of the light receiving neurons (photoreceptor) and vision decline. Clinically, AMD is divided into two subgroups: dry or atrophy form and wet or exudative form. Twenty percent of AMD patients have the wet form. The wet AMD is linked to choroidal neovascularization located in the subretinal macular region, with subsequent bleeding, and a possible sudden loss of central vision. AMD is an incurable devastating disease though the wet form is treatable by anti-vascular endothelial growth factor (VEGF) drugs to inhibit choroidal neovascularization so as to improve or maintain patients' visual function. For the dry form of AMD unfortunately, there is no effective treatment available in the clinic though the light at the end of tunnel is emerging: cell-based therapy is a potential solution for treating AMD (Chichagova et al., 2018).

To understand cell-based therapies for AMD, we need clarify their scientific rationale. Mammalian retinas are composed of multiple layers of retinal neurons and nurtured by two circulating systems: photoreceptors in the out nuclear layer (ONL) are fueled by the choriocapillaris across the RPE whereas all other retinal cells are supported by the optical nerve artery (Chichagova et al., 2018). It is clear now that the photoreceptor rod with a longer outer segment (OS) directly uptakes glucose by its cell surface transporter Glut1 through cell-cell contact with the RPE that passes nutrients from the underneath choriocapillaris (Wang et al., 2016). Rod surface contact with the RPE is required for glucose release to the subretinal space for the photoreceptor cone to take up (Gospe et al., 2010). Without the glucose supply from the RPE, impaired by the underneath drusen deposition, rods initially, and then cones will die of starvation (Wang et al., 2016). Apparently, glucose subretinal injection is not a viable long-term therapeutic option for retinal degeneration patients; thus, cell transplant can be an alternative, supplying the retina with not only neuroprotective and neurotrophic substances including glucose and cell-cell contact with the RPE, but also possibility of functional replacement of lost photoreceptor and RPE cells (Wang et al., 2016, 2018). Based on this assessment, an ideal stem cell source for AMD treatment in clinical trials is thought to exhibit two properties: it can expand towards a correct mature stage with limited potential *in vitro* and can functionally integrate into both the neuroretina and the RPE upon transplantation. Unfortunately, no such a stem cell with the dual-potential to differentiate into both RPE and photoreceptor cells *in vivo* has ever been identified in mammas. Alternatively, pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) derived from the inner cell mass of a blastocyst and induced pluripotent stem cells (iPSCs) created by forced expression of several stem cell genes in somatic cells like fibroblasts, can differentiate into either RPE or photoreceptor cells *in vitro* (Lamba et al., 2008; Sharma et al., 2019). The resultant tissue-specific cells can integrate into the RPE or the neuroretina in model animals after subretinal transplantation to functionally rescue or slow their visual deterioration (Lamba et al., 2008; Sharma et al., 2019). This is a typical cell replacement therapy model to intend to "cure" retinal degeneration. However, both RPE and photoreceptor cells need be replaced simultaneously to cure AMD patients, and no such a retinal progenitor-like cell (RPC) has ever been identified during PSC differentiation *in vitro*, which would be directly transplanted into lesion sites and able to give rise to both RPE and photoreceptor cells *in vivo*. In practice, in most clinic trials, PSCs, often iPSCs of patient's origin that can avoid host immune rejection upon transplantation and has no ethic issue compared to ESCs, are first directed to differentiate into RPE *in vitro* and then transplanted subretinally to improve AMD patients' vision. No clinic trial desired to transplant PSC-derived photoreceptor or combination of both photoreceptor and RPE cells has been reported because functional integration and long-term survival of grafted photoreceptors are extremely challenging given that inflammation, a hallmark of AMD, intends to provoke immune rejection. Most ongoing clinical trials now focus on the non-replacement strategy, i.e. the grafted cells, mostly RPE cells derived from iPSC, are subretinally delivered to repair the RPE and to provide nutrients and neuroprotective substances to the remaining photoreceptors to slow down and/or to prevent them from further degeneration (Chichagova et al., 2018). This non-replacement strategy is not intended to cure the disease by replacing lost retinal neurons, but instead, is targeting those still functioning photoreceptors, particularly those leftover cones for the central vision (Wang et al., 2016). Another common cell source for non-replacement cell therapy is mesenchymal stem cell (MSC), mostly bone marrow stromal cell (BMSC) (Chichagova et al., 2018). Compared to the PSC-derived RPE cell, it is much easier to have sufficient well-qualified and patient-specific BMSCs for immediate transplantation with no immune rejection problem. However, neither can BMSCs differentiate into RPE/photoreceptor cells nor integrate into the retina/RPE; they only stay in the subretinal space between the ONL and the RPE to secrete some neuroprotective factors such as VEGF to slow down photoreceptor degeneration for relatively short period of time before they die of starvation and then are phagocytosed by macrophages and/or RPE cells. Apparently, RPE cell is therefore the best cell source for treating AMD at present.

To manufacture patient-specific iPSCs for differentiation into mature RPE cells is a labor-intensive and time-consuming process. Although after directed differentiation it is relatively easy to enrich for iPSC-derived RPE cells to minimize undifferentiated and extremely tumorigenic iPSC, it is still very difficult to localize viral vector random insertions of the stemness genes used for making iPSCs, which may cause unfavorable genetic mutation(s) and lead to a unknown consequence upon transplanted to patients. Alternatively, small number of healthy RPE cells can be isolated from AMD patients for transplantation back to the lesion site of the patients (Chichagova et al., 2018). The major problem for this alternative is to expand these primary RPE cells *in vitro*. To solve this problem, we launched a series of investigations to make mammalian RPE cells regain their self-renewal and neuron transdifferentiation capacities by our novel sphere-induced reprogramming protocol that immortalizes murine RPE cells and transforms them to RPE stem-like cell (iRPESC) with dual-potential to differentiate into cells expressing either RPE or photoreceptor markers *in vitro*. iRPESCs are the RPC-like cells that do not need to be directed to a photoreceptor or RPE cells before transplantation and are able to differentiate into both photoreceptor and RPE cells simultaneously at the graft sites and integrate into the related tissues, the ONL and RPE, respectively (**Figure 1**) (Chen et al., 2020).

It is well known that in amphibians, and to some degree in embryonic birds, not only can their RPE cells proliferate *in vivo* but also transdifferentiate to restore retinal neurons following retinal injury (Haynes and Del Rio-Tsonis, 2004; Lamba et al., 2008). This process in amphibians is highlighted by initial transition of RPE cells to RPCs that migrate away from the RPE and lose cell-cell contact, they proliferate to form 3D clusters where they transdifferentiate into new photoreceptors and other retinal lineages. Many adult stem cells like BMSCs are found in 3D clusters within hypoxic niches in the body, which are critical for maintaining their viability and differentiation potential (Tsai et al., 2012). Mechanistically, induction of stem cell reprogramming factors such as Oct4, an essential stemness gene for iPSC, by

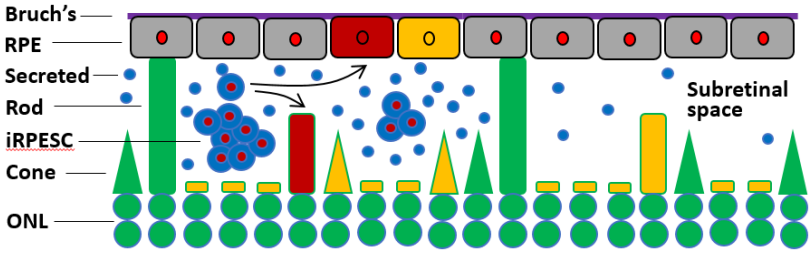

Figure 1 | **A diagram of iRPESC aggregation/migration in the subretinal space, and transdifferentiation/integration into both the RPE and the ONL after subretinal transplantation.** Yellow color indicates those degenerated RPE and photoreceptor cells whereas red color indicates that iRPESCs (blue color) are transdifferentiated into either RPE or photoreceptor cells, and that the transdifferentiated cells are integrated into the RPE and ONL, respectively. The blue dots indicate those neuroprotective and neurotrophic substances that iRPESCs secrete *in vivo* to prevent relatively healthy RPE and photoreceptor cells from further degeneration, and to maintain their viability. iRPESC: Sphere-induced RPE stem cells; ONL: out nuclear layer; RPE: retinal pigment epithelium.

the hypoxia transcription factor Hif1a leads to induction of the DNA methyltransferase Dnmt in these niches, which in turn methylates CpG sites in the promoters of the cyclin-dependent kinase inhibitor Cdki such as P16 and P21 to stably silence these genes and thereby ensure stem cell renewal ability (Tsai et al., 2012). Unlike amphibians, mammalian RPE cells have lost such a capacity to regenerate photoreceptors. Our ability to successfully manufacture iRPESC is a testimony to the fact that mammalian RPE tissues retain their developmental signatures in their genomes and that they have two potential developmental lineages: 1) to produce RPE cells, and 2) to transdifferentiate into other retinal cells when exposed to certain intrinsic/extrinsic cues. Sphere formation in culture mimics the 3D hypoxic niche of adult stem cells, and is widely used for maintenance and propagation of these cells in culture.

Recent studies from our group and others demonstrate that hypoxia within spheres activates Hif1a and cytoskeletal mechanosignaling changes in this 3D environment triggers Hif1a superinduction, which is required along with hypoxia for Hif1a to reach a threshold level for binding the *Oct4* gene promoter, whose induction leads to expression of Dnmt and stable silencing of Cdki. The result being that even transient exposure to such a 3D environment promotes long-term cell proliferation capacity. Such stable silencing of Cdki is required for iPSC reprogramming. Beyond regulation of Cdki, Hif1a and stem cell transcription factors such as Oct4 collaborate to induce pathways that converge to epigenetically mark sets of genes that drive lineage differentiation (Harikumar and Meshorer, 2015). These changes reflect a switch from all repressive H3K27me3 marks, to bivalent marking where activating H3K4me3 nucleosome inhibitory marks co-exist with H3K27me3 repressive marks (Harikumar and Meshorer, 2015). This bivalency pathway is induced by hypoxia and cytoskeletal mechanosignaling and it also drives reprogramming of somatic cells to a stem cell phenotype during 3D tumor outgrowth. Similarly, in the early sphere-like embryo, hypoxic induction of stem cell factors, Dnmt and in turn regulation of Cdki expression controls the onset of regional differentiation (Tsai et al., 2012). We demonstrate that transient 3D culture induces the bivalency pathway in mouse RPE, and these cells stably gain the ability to transdifferentiate into photoreceptors in culture, and they generate functional photoreceptors that contribute to visual acuity following subretinal transplant in mouse models of retinitis pigmentosa and AMD (Chen et al., 2020).

In summary, we developed a protocol to establish a sphere-induced cell reprogramming process for mammalian RPE cells to regain renewal capacity and transdifferentiation potential as with RPC-like cells and to be transplanted without directed differentiation, to the subretinal space where they can stay, migrate, transdifferentiate, and integrate in nearby tissues (**Figure 1**) (Chen et al., 2020). When regaining RPE properties by default and integrating into the RPE tissue, the grafted iRPESCs are supposed to functionally replace lost/damaged RPE cells in support of remaining ONL neurons for a long term. When staying in the subretinal space, the non-integrated iRPESCs may secrete large amounts of paracrine factors, particularly VEGF to protect the retinal neurons from further degeneration for a short term (**Figure 1**) (Liu et al., 2020). The potential of iRPESC to transdifferentiate into photoreceptors is still very limited particularly *in vivo*, the detail intrinsic and extrinsic factors for such a neural transdifferentiation is not known; but, as soon as these factors are finally identified, effective treatments for AMD will be fully formulated for a real breakthrough. The iRPESC work is based on mouse system and the conclusions may not be proven in human system, more works are clearly required to avoid any misinterpretation and pre-assumption.

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