Retinal Ganglion Cells Regeneration

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Glaucoma, the second leading cause of blindness worldwide, is an incurable neurodegenerative disorder due to the dysfunction of retinal ganglion cells (RGCs). RGCs function as the only output neurons conveying the detected light information from the retina to the brain, which is a bottleneck of vision formation. RGCs in mammals cannot regenerate if injured, and RGC subtypes differ dramatically in their ability to survive and regenerate after injury. Recently, novel RGC subtypes and markers have been uncovered in succession. Meanwhile, apart from great advances in RGC axon regeneration, some degree of experimental RGC regeneration has been achieved by the in vitro differentiation of embryonic stem cells and induced pluripotent stem cells or in vivo somatic cell reprogramming, which provides insights into the future therapy of myriad neurodegenerative disorders. Further approaches to the combination of different factors will be necessary to develop efficacious future therapeutic strategies to promote ultimate axon and RGC regeneration and functional vision recovery following injury.

retinal ganglion cell ESC iPSC organoids reprogramming

1. Generation of RGCs from Stem Cells

1.1. ESCs

Mammals, unlike teleost fish, cannot regenerate the retina after various types of injury. Consequently, numerous in vitro protocols of RGC differentiation from pluripotent stem cells were created [1][2]. Although the supposedly mammalian adult retinal stem cells were found in the pigmented ciliary margin [3], there is no irrefutable evidence that they are able to regenerate RGCs damaged by injury or disease, which is in contrast to the situation in zebrafish and amphibians. In the chicken, Fischer and colleagues found that RGCs could be induced from retinal margin cells of post-hatch chicken by the co-injection of insulin and FGF2 into the vitreous chamber [4].

bFGF-induced ESCs were able to generate RGC-like cells upon differentiation, which were capable of integrating into the host retina [5]. In addition, hESCs can be directed to retinal progenitors by a combination of Noggin, Dkk1, and IGF1, which subsequently differentiated primarily into ganglion and amacrine cells [6]. Another protocol adapted from previous work [6] extended the culture duration of the embryoid body and used 10% knockout serum replacement, resulting in an enriched population of functional RGCs from hESCs [7]. Sluch et al. described a protocol that led to the differentiation of hESCs to RGCs and their subsequent isolation [8], benefiting from a modified photoreceptor differentiation protocol [9]. Together with a novel Brn3b-tdTomato-Thy1.2 reporter line, they designed an original protocol called DIDNF+D (Dorsomorphin + IDE2 + Nicotinamide + Forskolin + DAPT) that can improve the efficiency of the differentiation and purification of stem cell-derived RGCs [10].

1.2. iPSCs

The establishment and development of iPSCs [11][12][13][14][15] and organoid culture systems [16][17][18][19][20], which mimic organogenesis in vitro, hold a great promise for a range of biological and biomedical applications, especially for regenerative medicine, by removing the limitation of replacement therapies and by enabling the development of in vitro disease models for drug screening. As an excellent cell resource, patient-derived iPSCs allow the regeneration of different and sufficient quantities of autologous cell types almost without the risk of immune rejection and iPSCs have already been generated from patients with multifarious diseases [21]. In addition, the procedure to generate urine-derived iPSCs with high reprogramming efficiency has been established [22], which provides a promising noninvasive source of stem cells and can subsequently differentiate into desired cell types.

By mimicking RGC genesis, Deng et al. performed a stepwise and efficient differentiation of human Tenon's capsule fibroblasts-derived iPSCs toward RGC-like cells by combining DLN (Dkk1 + Lefty A + Noggin) treatment and Atoh7 overexpression sequentially [23]. iPSCs can differentiate into RGCs in neural induction and retinal differentiation culture medium [24]. The overexpression of a single gene can achieve the same effect. Mouse ESCs or iPSCs were able to be induced into RGCs by Pax6 overexpression and subsequent limiting-dilution culture [25] [26]. In addition, retrovirus-mediated Neurod1 overexpression in iPSCs together with retinoic acid and taurine treatment increased the expression of RGC markers [27]. Similar to this, a single chemical, DAPT, can induce Pax6/Rx-positive stem cells to undergo differentiation into functional RGCs [28].

However, the potential risks of iPSCs such as genomic instability and immunogenicity differences [29][30][31][32] cannot be ignored. Whilst this might seem to be a truism, it is nevertheless a crucial problem to address.

1.3. Organoids

Since 3D retinal organoids were successfully established from mouse [19] and human ESCs [33], a number of adapted protocols were established for specific research purposes, such as generating retinal organoids from iPSCs [34][35], the formation of specific structures [35][36][37], or the formation of cell-specific features [9][36][37][38]. This suggests a possibility to restore vision via the transplantation of RGCs gained from retinal organoids because the efficient derivation of sufficient numbers of functional and integration-competent cells might partly remove a key limitation for regenerative medicine. Indeed, cells from ESC-derived eye-like structures were integrated into the RGC layer and differentiated into neurons when transplanted into adult eyes [39]. Moreover, Tanaka et al. efficiently generated self-induced RGCs with functional axons from mouse and human iPSCs by combining the cultivation of 3D floating aggregates with a subsequent 2D adhesion culture [40][41].

2. Generation of RGCs by Reprogramming

Nowadays, more and more groups aim at directly reprogramming fibroblasts, MG, or other somatic cells into retinal neurons in vivo, and some progress has been made. Mammalian MG cannot be maintained in dishes for a prolonged period, but they can be considered as the endogenous stem cell-like cells, which can be reprogrammed

into bipolar, amacrine, and ganglion cells under certain conditions [42][43][44][45][46][47][48]. For example, combining repressing Notch signaling with activating TNFα signaling can stimulate MG proliferation to generate neuronal progenitor cells that subsequently differentiate into retinal neurons [49].

There is no doubt that TFs have played an essential role in the field of cell reprogramming and this will continue. Klf4, which can promote ESC self-renewal [50], is well known as one of the four famous Yamanaka factors [11]. Although Klf4 functions as a transcriptional repressor for axon growth of RGCs and other CNS neurons, it is also a potential candidate factor for reprogramming to replenish RGCs. Rocha-Martins et al. demonstrated that Klf4 was sufficient to change the potency of lineage-restricted retinal progenitor cells to generate RGCs in vivo [51].

Another attractive TF, Ascl1, displays a magic ability in cellular reprogramming in the retina. It is capable of reprogramming mouse MG into bipolar and amacrine cells in vitro [52]. The forced expression of this neurogenic TF in MG gave rise to amacrine, bipolar, and photoreceptor cells in young mice after NMDA treatment [48]. In addition, the same group found that MG-specific overexpression of Ascl1, together with NMDA and trichostatin-A, enabled mice to regenerate functional retinal interneurons [42]. Judging from previous data, single Ascl1 is not sufficient to convert MG into RGCs either in vitro or vivo [42][48][52]. Meanwhile, Meng et al. found that adenovirus-mediated transduction of Ascl1, Brn3b(Pou4f2), and Ngn2 can directly convert mouse fibroblasts to RGC-like cells [53]. Recently, Xiao et al. reported that a combination of triple TFs Ascl1, Brn3b/3a, and Isl1 not only reprogrammed fibroblasts into self-organized and networked sensory ganglion organoids but also induced RGCs [54]. Subsequently, Wang et al. confirmed that this cocktail treatment worked well in inducing RGC-like cells [55]. It should be emphasized that strictly speaking, the RGC-like cell is a more proper term for RGCs induced in vitro, because previous studies have shown that it is difficult to distinguish RGCs from peripheral sensory ganglion neurons, since both of them share many common molecular hallmarks. This may no longer be an issue, as our group has recently found that a combination of Pax6 with Brn3a or Brn3b can serve as a unique identifier for RGCs [54].

Apart from Klf4 and Ascl1, Atoh7, an essential basic-helix-loop-helix TF for establishing RGC fate, plays a vital role in RGC regeneration. The forced expression of Atoh7 promotes the differentiation of MG-derived retinal stem cells into RGCs [44][56]. In addition, Dkk1 + Noggin + DAPT and the overexpression of Atoh7 together could convert fibroblasts into RGCs [57]. Furthermore, Neurod1-expressing amacrine and photoreceptor progenitors can be reprogrammed into RGCs when Atoh7 is inserted into the *Neurod1* locus [58]. Xiao et al. showed that combining Atho7 with Brn3b was able to reprogram mature mouse MG into RGCs efficiently and the MG-derived RGCs were functional, made appropriate central projections, and improved visual responses [59]. Ngn2 alone is also sufficient to lineage, reprogramming postnatal mouse MG into RGC-like neurons in vitro and inducing the generation of this neuronal type from late retinal progenitors in vivo [43].

Researchers' ambitions are unbounded. Numerous modified methods were created to achieve the goal of mature somatic cell-to-neuron conversion. iPSCs were generated from fibroblasts via mRNA reprogramming and subsequently differentiated into a retinal fate by modifying a previously established protocol in a directed, stepwise manner [60]. Most recently, Lu et al. have shown that the ectopic expression of Oct4, Sox2, and Klf4 in RGCs can

restore youthful DNA methylation patterns and transcriptomes, promote axon regeneration after injury, and reverse vision loss in models of glaucoma and aged mice [61]. Perhaps, other TFs and epigenetic modifications may also be involved in this process or RGC regeneration. Aside from TF overexpression, knockdown of the RNA-binding protein Ptbp1 by the CRISPR-CasRx system also converted MG into RGCs in mature murine retinas, which alleviates the symptoms associated with RGC loss [45]. Moreover, this approach can also make glia-to-neuron conversion in the brain [45].

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