IDENTIFYING RELIABLE SOURCES OF STEM CELLS FOR CELL REPLACEMENT THERAPY IN RETINAL DEGENERATION DISEASES

Eloísa Herrera González de Molina
Institut de Neurociències d'Alacant
Marta Agudo Barriuso
Instituto Murciano de Investigación Biosanitaria Virgen de la Arrixaca

1. Summary

Traumatic, hereditary or acquired degeneration of retinal neurons are common causes of visual impairment. Cell replacement therapies may in the future be an approach to restore visual function in individuals with these conditions. In this project we use mouse models to identify putative sources of cells that could be safely used for retinal degeneration conditions.

Hypothesis

The retina is part of the central nervous system and diseases or traumas that occur with neuronal death cause loss of vision. To date, there are no therapeutic strategies to replace dead neurons or, from a less ambitious point of view, to delay or stop their degeneration. Our hypothesis was based on previous research proposing the cells of the ciliary margin of the retina, located in the peripheral zone of the eye, as a potential source of protective factors that would slow neuronal death in degenerative diseases of the retina such as glaucoma, which causes the progressive loss of retinal ganglion cells (RGCs), retinitis pigmentosa or macular degeneration, which cause the loss of photoreceptors, or that could be used as a source of cellular replacement in these pathologies.

Specifically we aimed:

1. To determine whether factors secreted by neural stem cells from the peripheral mammalian retina would have a neuroprotective effect that delays or stops neural degeneration in the retina.

2. To elucidate whether neural stem cells from the peripheral mammalian retina have the potential to differentiate in vivo so that they can be considered a reliable source of cells to replace damaged neurons in retinal degenerative diseases.

2. Results

Ciliary body cells (CCCs) are a cell population located at the peripheral region of the retina that has been proposed as a potential source of cells to be used in neuroprotection and/or replacement therapies in retinal degenerative diseases. The objective of this project was two-fold: First, to investigate a possible neuroprotective effect of these cells and, second, to evaluate their potential capacity in neuronal replacement in vivo and compare it with other types of neural stem cells such as those obtained from the subventricular zone (SVZ) whose potential has been previously tested in other tissues. The CCCs are a very small cell population and therefore, if these cells turn out to have regeneration capacity, it will be essential to know the possibility of being expanded in vitro in order to get enough for their injection into the damaged eye. For this reason, in addition to testing the potential of CCCs to replace damaged cells, we have amplified these cells in vitro by generating neurospheres (N-CCC) and compared their neuroprotection and replacement potential with primary CCCs. Therefore, three types of cells have been injected: i) CCCs newly isolated from the ciliary body (primary), ii) CCCs from neurospheres (N-CCC) amplified in vitro and iii) neural pluripotent cells obtained from SVZ. Each cell type has been compared its their respective control, which are the media in which they are suspended.

In order to address the questions initially proposed in this project (potential of CCCs for neuroprotection and/or replacement), we have performed the following assays:

- Neuroprotection assay: To analyse a possible neuroprotective effect of CCCs the cells were injected into the retina at the same time as we performed axotomy of the optic nerve (ON), which induces progressive degeneration of RGCs. Previous data from Dr Agudo-Barriuso's laboratory have shown that at 5 days post-axotomy 50% of the RGCs have degenerated, at 14 days 12-15% survive and at 45 days only 1-2% of the original population of the RGCs survive. Therefore, we quantified the survival of RGCs at these three times (5, 14 and 45 days).

- Replacement assay: To study the potential of these cells to differentiate into neurons paving the way for a putative replacement of retinal cells, we performed two types of assays:

i. Differentiation and degeneration at the same time: We performed cell injection at the time that induced degeneration of RGCs by ON axotomy. At 14 and 45 days we analysed the ability of the injected cells to differentiate into neurons.

ii. Differentiation after degeneration: Cellular injections are performed in retinas in which the RGCs have already degenerated (45 days after axotomy). In this way, we avoid the possibility that the toxic factors and reactive glia that the degenerating RGCs may be producing could affect the differentiation capacity of the injected cells.

Neuroprotection assay

We injected the three types of cells and their controls (their respective resuspension medium) after performing ON axotomy and quantified the survival of RGCs at different times after injury (from 3 to 45 days). Our results show that, in retinas treated with CCC, N-CCC or SVZ, the survival of RGCs is significantly higher than after treatment with their corresponding vehicles and that this neuroprotective effect is proportionally higher at longer times after axotomy (Figure 1).

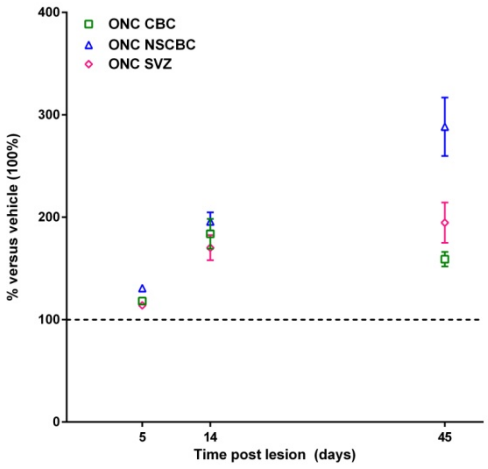

Figure 1. Neuroprotection

Graph of percentage (± sem) of survival of axotomised RGCs in retinas treated with different cell types over time. The number of surviving RGCs in the respective control groups (ONC + vehicle) has been considered 100%. n = 4-6 retinas per time and cell type.

Replacement Assay

i) Differentiation and degeneration at the same time

In experiments in which we perform injections of CCC, N-CCC and SVZ while performing ON axotomy, we also analyse the fate of the injected cells at 14 and 45 days. The three types of cells were obtained from an actin-GFP mouse line to visualize their location and morphology in the injected retinas. Both at 14 and 45 days post-transplantation, the three cell types formed an epiretinal net into the analysed retinas but integration of fluorescent cells into the retina was not observed in any case (data not shown).

ii) Differentiation after degeneration

After the negative results of the previous assay, we thought that the pro-inflammatory environment resulting from the massive death of degenerating RGCs and the consequent activation of the microglia could generate a hostile environment, not very permissive for the migration and integration of the injected cells. For this reason we decided to transplant the cells once the RGCs have degenerated and disappeared completely, i.e. 45 days after the axotomy. The retinas were analysed 30 and 90 days after transplantation (i.e. 75 or 105 days after axotomy).

Both 30 and 90 days after transplantation of each cell type, epiretinal nets containing GFP-positive cells were observed (Figure 2a-c). In addition to the net, we observed GFP cells into the innermost layer of the retina where the RGCs were located prior to their degeneration. Many of these GFP cells expressed NeuN, a marker for differentiated neurons. In the case of SVZs, GFP signal was also observed in the internal nuclear layer (Figure 2C).

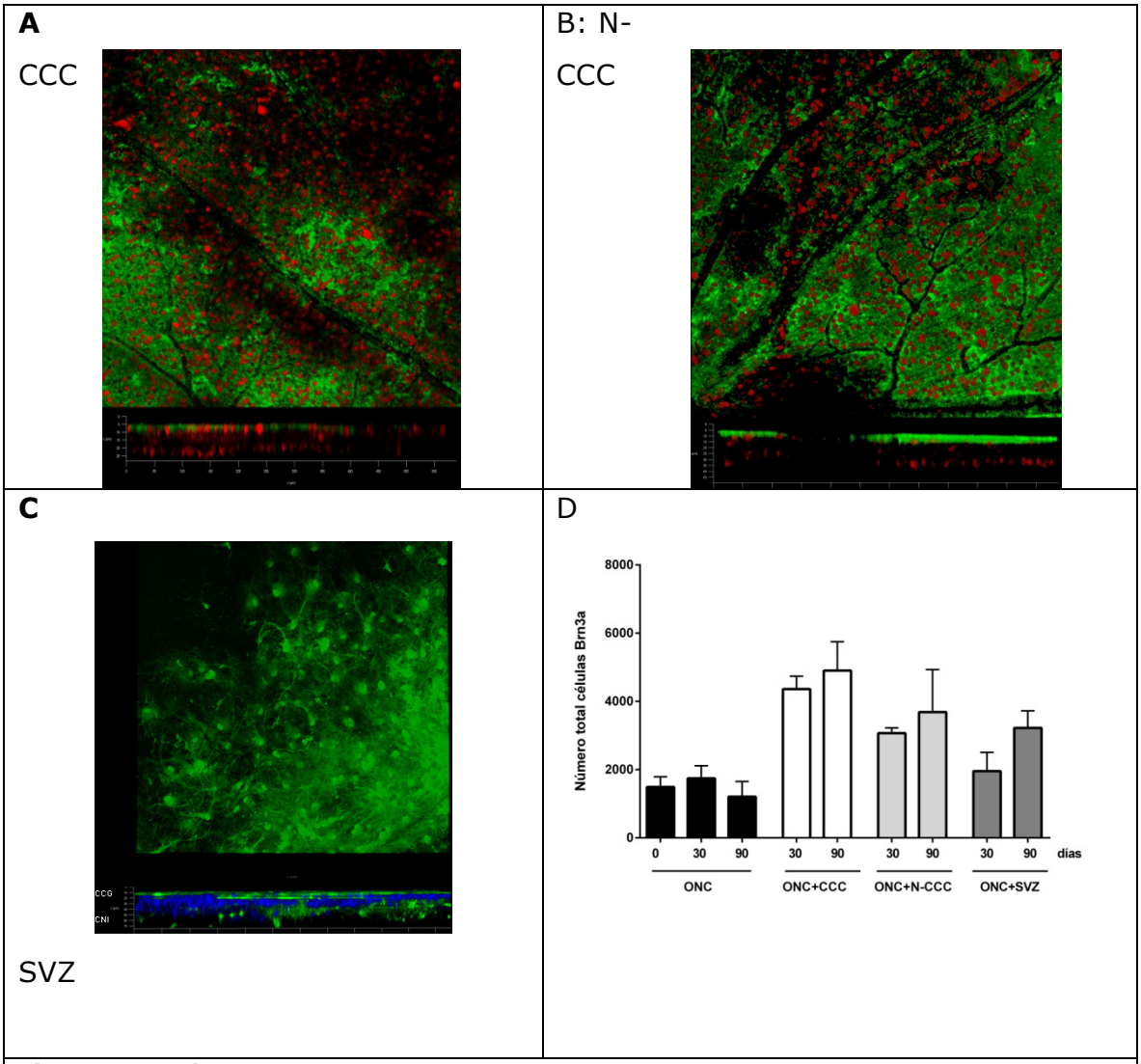

Figure 2. Replacement

(A-C) Confocal images showing in green cells CCC-GFP, N-CCC-GFP and SVZ-GFP and in red NeuN positive nuclei, in the retina 90 days after transplanting them in retinas depleted of RGCs. Above view of the ganglion cell layer. Below, section of the retina. CCG: ganglion cell layer, CNI: internal nuclear layer.

(D) Total number ± standard deviation of Brn3a + cells quantified in control retinas depleted of RGCs (ONC) 45 days before or depleted of RGCs 45 days before and treated with each cell type (ONC + CCC, ONC + N-CCC, ONC + SVZ).

Finally, when comparing the total number of RGCs in control retinas (axotomy without transplantation) with that of transplanted retinas, we observed a significantly higher number of cells expressing specific RGCs markers such as the Brn3a transcription factor in depleted retinas (Figure 2D) suggesting that transplanted cells have differentiated and express RGCs markers.

In summary, our data suggest that: 1) cells isolated from the peripheral adult retina, cells derived from them amplified in vitro and cells from the subventricular zone, when injected intravitreally into the retina, modestly delay the degeneration of RGCs damaged by axotomy of ON and 2) When these cells are injected into retinas depleted of RGCs, they begin to express neuronal markers, suggesting that they may differentiate to neurons and therefore open the door to their use as a possible source of cells in replacement therapies in the future.

3. Relevance of possible future implications

Today, the treatment of neurodegenerative diseases is one of the greatest challenges in medicine. The increasing development of cell therapy approaches has opened up new hopes for repairing damage in different tissues. Lesions in the central nervous system are particularly challenging given the extreme complexity of neuronal circuits. Neuronal degeneration is a long-term process and therefore, cell therapy in the nervous system could be useful in two possible scenarios: 1) transplanted cells preventing neuronal degeneration in the host tissue (neuroprotection) and 2) transplanted cells differentiating and acquiring the functional properties of degenerated cells (replacement).

Thanks to the superficial location of the retina, cell therapy applied to the retina is the one that is most likely to succeed. In fact, transplantation of stem cells into damaged retinas is currently underway with phase I/II clinical trials. However, results obtained to date, using autologous stem cells derived from bone marrow in patients with a high degree of degeneration who were practically blind, have not demonstrated an improvement in vision and in some patients a worsening has even been described. In animal models, intravitreal transplantation of stem cells not derived from the CNS, e.g. mesenchymal stromal cells, produces a glial and inflammatory response in the retina that deforms its laminar structure. These results, both in patients and in animal models, suggest that the use of totipotent stem cells in cell therapy is not recommended.

In this project we have explored the neuroprotective potential and the differentiation capacity of two types of neural cells, cells from the ciliary margin of the adult retina (CCC), pluripotent cells with a more restricted differentiation capacity than totipotent

cells, which have been proposed as a good option because they are the natural source of stem cells in the retina of non-amniote vertebrates. In addition, we have compared the potential of these cells with those of non-retinal stem cells derived from the subventricular zone (SVZ) that have previously been used for replacement trials in other contexts. Our data show that unlike multi- or totipotent stem cells, pluripotent neural stem cells do not produce an inflammatory response in the retina, nor are they toxic, resulting in improved safety during treatment of patients. In addition, we have observed that both primary cells (CCC, SVZ) and those amplified in culture from them (N-CCC) have long-term neuroprotective capacity over long periods and that, under certain conditions, they may even have the capacity to express specific markers of differentiated neurons. The neuroprotective impact of these pluripotent neural cells on retinal neurons during the process of degeneration has several positive implications: On one hand, these cells could be used in the future to delay the process of neuronal death in chronic degenerative diseases of the retina such as glaucoma. On the other hand, it seems possible to keep them in culture remaining bioactive, which opens the door to generate biobanks of this type of cells. In addition, the fact that pluripotent neural cells transplanted into animal models depleted of RGCs express specific markers of mature neurons suggests that these cells could end up differentiating and integrating into the circuit. In spite of these hopeful experiments, in order to obtain a clear conclusion and start thinking about a possible clinical application, more analysis are needed to confirm that cells that express RGCs markers actually come from the injected cells. This promising result suggests the possibility of treatment even after neuronal loss, and would broaden the range of treatments. In the case of RGCs, this is certainly a remote possibility because the connection of these cells with the brain involves the regulation of a set of axonal guidance events that are not activated in the adult individual. However, in the case of other types of retinal neurons such as photoreceptors, horizontal cells, bipolar cells, etc, our results open a more plausible hope.

4. Papers generated from the Project

During the development of this project we have acquired a broad knowledge on the biology of mammalian ciliary margin cells which we have reported in high impact journals:

- Marcucci F, Murcia-Belmonte V, Wang Q, Coca Y, Ferreiro-Galve S, Kuwajima T, Khalid S, Ross M.E., Mason C, and Herrera E* (2016) The Ciliary Margin Zone of the Mammalian Retina Generates Retinal Ganglion Cells. ***Cell Reports*** 1 (12) 3152-3164. Cover caption. Impact factor: 8.1.

- Fernández-Nogales M*, Murcia-Belmonte V*, Yu Chen H, Herrera E* (2018). The peripheral eye: A neurogenic area with potential to treat retinal pathologies? ***Prog Retin Eye Res.*** 2019 Jan; 68:110-123. doi: 10.1016/j.preteyeres.2018.09.001. Impact factor: 11.8.

Results detailing the data described in this report, which were the subject of the initial proposal, are being finalized and will be sent shortly for publication.