Editorial Commentary

Pluripotential stem cells as replacement therapy in degenerative diseases of the eye

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Loss of vision has devastating effects on the quality of life of both individuals and their caregivers as well as causing a huge economic burden to families and society. It has been estimated that by 2020 there will be almost 200 million people worldwide losing sight because of dying photoreceptors in age related macular degeneration (AMD). Intense efforts are underway to develop therapies that can prevent or block photoreceptor loss in AMD. These efforts include lifestyle changes, nutritional supplements and neuroprotective agents, but to date they have had only marginal success. These approaches also do not help the growing numbers who have already lost photoreceptors. Another major line of research to help those patients, and the one reviewed thoroughly by Gagliardi *et al.*, (1) is to develop transplantable photoreceptors that can integrate into the retina, restore light sensitivity and, hopefully, restore vision.

Retinal, and particularly photoreceptor, transplantation in mammals has been studied for over 70 years. In the 1980's and 1990's a number of groups showed that grafts of retina, including embryonic human retina, could survive and develop in host eyes (2-4). Over the next decade many of the major retinal research groups studied the possibility of transplant therapy, and most reached the same conclusions. While cells could survive as grafts for several months, there was little evidence for true integration into host tissue and immature photoreceptors did not mature into fully functional structures (5,6). These early studies also could not answer the dominant question of where sufficient quantities of graft tissues could be obtained to help patients.

The largest need for transplants in AMD is for the two cell types most affected, namely cone photoreceptors and retinal pigment epithelium (RPE). On the other hand, most research on retinal transplantation has focused on the rarer group of inherited rod photoreceptor degenerations collectively known as retinitis pigmentosa (RP). RP affects about 1 in 4,000 people in the US and Europe and probably similar numbers in the rest of the world. Over 60 genes have been identified as causative for RP, with autosomal dominant, autosomal recessive and X-linked inheritance patterns (7). RP causes loss of rod photoreceptors, leading to night-blindness, and a secondary loss of cone photoreceptors eventually leading to complete loss of vision. A major reason for the focus on therapies for RP has been that excellent mouse models of the disease exist, including mutations in many of the genes that cause the human disease. Even with the availability of inbred mouse strains transplantation studies have shown only modest success. Transplantation of differentiated rods leads to poor survival and integration; transplantation of early tissue leads to the formation of self-contained mini-retinas. Neither outcome is ideal for restoration of vision. In addition, while useful for mechanistic studies, the work in mice has clearly not been applicable to human since no equivalent source of tissue is available.

The tissue source issue has been overcome with the introduction of stem cell lines, particularly induced pluripotential stem cells (iPSCs) that can be produced from adult tissues. Gagliardi *et al.* present a comprehensive review of the current efforts to obtain and use transplantable

photoreceptors through the controlled treatment of stem cells. The advantages of a dividing stem cell pool are well known and include and essentially unlimited supply of cells and the possibility of deriving iPSCs from patients and thus avoiding graft rejection problems. However, there are still many problematic issues with this approach and these are discussed extensively by Gagliardi *et al.*

A very useful early part of their review is a description of retinal development focused on the spatial and temporal expression of transcription factors. The review makes a clear argument for multiple series of binary choices made by cells through biasing expression levels of mutually antagonistic transcription factors by the actions of exogenous growth factors. For example, Vsx2 delineates retina and microphthalmia-associated transcription factor (MITF) delineates RPE and these two molecules mutually repress each other's transcription. FGF signals from lens ectoderm promotes retina development (and Vsx2 expression) while extra ocular mesenchyme provides a source of transforming growth factor beta (TGF-β)/bone morphogenetic protein (BMP) molecules that promote formation of RPE (and MITF). This combination ensures that the front layer of the optic cup develops into retina and the back layer in RPE.

Similar interactions among transcription factors, and similar actions by growth factors, have been mapped at multiple stages of retinal development. So how does this relate to iPSCs? If all of these steps are essential to generate mature retinal photoreceptors, then taking an iPSC culture and turning the cells into photoreceptors may require an extremely complex series of treatments with growth factors in a specific sequence to achieve the desired result. For example, the TGF-β family member activin plays a key role at several stages in retinal development through regulation of SMAD transcription factors. These can suppress expression of progenitor genes and, when the transcription factor FOXH1 binds at an adjacent site, cause the activation of specific photoreceptor genes (8). The complexity of individual growth factors acting at multiple times, and their action modulated by combinations of transcription factors, is something that will need to be understood to fully gain control of iPSC differentiation. There is an alternative approach that might help. Just as fully differentiated cells can be induced to become iPSCs by transfection of just four genes, there is the hope that many steps in retinal development can be short circuited by application of specific transcription factors. The necessary factors, or even whether this is going to be possible, have yet to be identified.

While it would be ideal to have a culture of iPSCs behave synchronously and all cells develop into the desired cell type, this does not happen. Due to a combination of inherent asynchronous behavior and cell-cell interactions among the population, cultures tend to form complex structures of multiple cell types. This has led to a variety of procedures to try and purify the cell types of interest, photoreceptors in this case. The most labor-intensive approach has been to allow cultures to develop as 3D organoids and then manually dissect the presumptive photoreceptor layer for future treatments. This is clearly not an approach that lends itself to the high throughput that will be necessary to meet the needs of the growing patient population.

One of the arguments made by Gagliardi *et al.* is that there is a need to find a way to produce and purify a photoreceptor progenitor population because these cells poised to undergo their terminal differentiation would be the most ideal for transplantation. Using mouse cells several groups have generated strains that express GFP under the control of a rod photoreceptor specific promoter. Thus, these cells will become fluorescent just as they begin differentiating into rods. This has led to some exciting results where multiple groups have claimed integration of fluorescent cells into the photoreceptor layer. Unfortunately, as Gagliardi *et al.* explain in some detail, many of these findings are suspect because there is now evidence for cytoplasmic transfer of fluorescent material between cells (9). The fluorescence observed in some of these reports may have been due to transfer of fluorescent protein into host cells rather than integration of graft cells. So where does that leave us? While it is now difficult to evaluate many of the published studies, new experiments using a variety of nuclear markers, such as Y chromosome or Barr bodies, are suggesting that some limited integration is possible.

Of course, without fluorescent markers there is a need for some other way of purifying photoreceptor progenitors. One surface marker that may fit this role is CD73 (ecto-5'-nucleotidase). This is an enzyme found in many tissues and plays an important role in both the immune system and many tumor cells. CD73 shows increased expression in mouse rod photoreceptor progenitors as they are beginning their terminal differentiation, and this can be detected using fluorescently labeled antibodies (10). Sorting the labeled cells gives a population that is highly enriched in photoreceptor progenitors. These cells are, however, fragile and some of the advantages of purifying them are lost by the trauma of sorting. Unfortunately, the expression of CD73 in human retinal cells is less clear and there are still conflicting results about whether it can be used to

isolate cells for transplantation. Clearly, identification of markers that identify cells poised to begin their terminal differentiation, and can be used to isolate these cells, are urgently needed because this appears to be the best stage for successful transplantation.

Transplantation involves surgery and there are many ongoing efforts to improve the technical aspects of the procedure. Typical transplantation involves inserting a needle into the subretinal space, injecting fluid to create a retinal detachment and then injecting the graft cells. Injecting a cell suspension provides the best opportunity to disperse the graft over a wide area and increase the chances for integration. The environment of the degenerating retina has several features that make it inhospitable for graft survival and integration. Even in the normal retina the outer limiting membrane (OLM) forms a physical barrier to cells integrating into their correct positions in the outer nuclear layer. Degeneration of photoreceptors leads to gliosis in Muller glial cells and the deposition of a number of inhibitory extracellular molecules. Controlled disruption of some of these barriers can significantly increase the integration of graft cells (11). Whether such an approach is suitable for a patient population with some remaining vision has yet to be decided.

The other transplantation technique discussed at length by Gagliardi *et al.*, is the use of sheets of cells. While this seems to help survival and differentiation of cells, the problem is that the purity is often lower and other retinal cells types develop and hamper normal integration. The active research in these surgical techniques suggests that improved transplantation protocols will soon be developed and this should help cell survival and integration.

So how do all these findings help patients going blind from AMD? Unfortunately, the answer is - not very much. Gagliardi *et al.* provide a clear review of what we know about directing cells into a cone photoreceptor differentiation pathway. So far this has proven to be more difficult than obtaining rods. We also need better models than the mouse to test the functional integration of cones. As a nocturnal species with a rod-dominant retina, it is not clear that the mouse retina can be sufficiently receptive to transplanted cones or provide the correct signals to help terminal differentiation of any cone progenitors. We even have a poor idea of how to define a cone progenitor to isolate and monitor cells.

While the above comments, and much of the Gagliardi *et al.* review, might seem to show lack of progress, it is important to realize that this is a relatively new field and

that progress so far has been extremely rapid. There is also one success story that needs to be emphasized. In addition to generating retinal neurons, iPSCs can be induced to produce RPE cells (12,13). Under the right growth conditions sheets of pigmented RPE can be detected and easily separated from other cells. Animal experiments showed that RPE transplants could rescue vision in a number of degeneration models (14-16). A number of small clinical trials have taken place and the grafts seem to be well tolerated and prevent further loss of vision (17,18). Because these early studies have been carried out on late stage disease, it is difficult to decide whether if the treatments were given to earlier stage disease patients they would prevent further degeneration and preserve sight. The data so far look promising and RPE transplantation is likely to pave the way for other more complex forms of retinal transplantation.

Gagliardi *et al.* have given us a detailed and thorough review of an active and important area of research and clinical investigation. Based on their review we can ask how far have we come and how far do we have to go to realize the goal of transplantation as a routine therapy for generative blinding diseases. Can we produce a cell population that can be used to generate cells for transplantation? Here the answer is a clear yes. The evidence is overwhelming that iPSCs can be produced from multiple sources, including autologous cells from individual patients. Can iPSCs be manipulated to produce desired cell types such as rod or cone photoreceptors or RPE cells? Here the answer is a qualified yes. It is possible to generate cells expressing markers of rods, cones and RPE. However, it is not yet possible to routinely produce pure populations of cells, or even highly enriched populations without substantial time and effort. More work is needed to streamline methods to guide the differentiation pathways of iPSCs and prevent unwanted cell types if this is to ever become a routine therapy. Can we define the best developmental stage to transplant cells? There is a growing consensus that committed progenitor cells, that is cells already destined to be rods or cones but not yet fully differentiated, show the best survival in grafts. Perhaps because they form a simpler epithelial sheet, it appears that more differentiated RPE cells can be successfully transplanted. Can transplanted progenitors or early stage differentiated cells complete their differentiation and integrate into host tissue? This remains one of the biggest remining questions. No one has yet managed to produce a fully mature photoreceptor in a graft. There is still much work to do in this area.

Similarly, the evidence for successful integration into host retina is still preliminary in even the well-controlled animal studies. There is still much to be done to understand the biochemical responses to degeneration (and aging) and the steps needed to provide a supportive environment for grafts. Can we restore vision with this approach? This is the ultimate question. To someone who is totally blind, the ability to perceive light and dark to navigate around obstacles is a huge improvement. It is very probable that this, and even some level of shape recognition, will be achieved within the next few years. To achieve higher acuity vision, it may be necessary to transplant cells into patients who still have a moderate level of vision. This raises a whole series of potential risks and ethical concerns that are beyond the biological topics covered in Gagliardi *et al.*

Overall, the review by Gagliardi *et al.* is an authoritative description and evaluation of the current state of the use of stem cells for retinal transplantation. From their article we can also see the areas where advances are needed to turn this procedure into a routine clinical treatment for an important series of diseases.

Acknowledgments

Footnote

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