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Cells previously identified as retinal stem cells are pigmented ciliary epithelial cells

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Background

Macular degeneration, diabetic retinopathy, glaucoma and retinitis pigmentosa are among the major causes of blindness and visual impairment. Currently available treatment strategies like laser photocoagulation, photodynamic therapy, anti angiogenesis therapy and drug delivery are ineffective in restoring the normal vision of retinal degeneration patients[1]. Resident stem cells in the eye have a potential for therapeutic use to regenerate and repair disease affected retina. The Ciliary Marginal Zone (CMZ) present in the retina of lower vertebrates like amphibians, fishes and birds is a source for retinal stem cells (RSCs) [2]. The stem cells present in the CMZ have been reported to play an important role in the post natal development and regeneration of retina in lower vertebrates. Extensive studies have been done on the regulation and maintenance of the CMZ stem cells[3]. The size of CMZ has decreased during the course of evolution from amphibians-reptiles-birds and is completely absent in mammals. The lack of CMZ is thought to be responsible for the inability of mammalian retina to regenerate after injury or degeneration. Ahmad *et al* and Tropepe *et al* reported for the first time in 2000 about the existence of a rare population of stem cells in a region of pigmented ciliary epithelium, an extension of retinal pigmented epithelium (RPE)[4,5]. These reports provided hope for the treatment of retinal degenerative diseases through transplantation or mobilization of these cells. The past decade has witnessed remarkable advances in the proliferation and differentiation capacity of ciliary epithelial (CE) stem cells and the factors affecting the maintenance of these stem cells. Proliferation and differentiation potential of CE stem cells has been studied in various species like rodents [6], porcine[7], rabbits [8], monkey[9]and humans[10] . These cells have also been tested for *in vivo* regeneration of retina in various retinal degeneration models. However, it was not clear that *in vitro* neurospheres originate from pigmented CE cells itself or involves RSCs or retinal progenitor cells residing in the CE before Cicero's *et al* report. In this report, authors have shown that neurospheres have been derived from differentiated pigmented ciliary epithelium and not involve retinal progenitor cells. Hypothesis of this study was to test the efficacy of CE derived stem cells for transplantation in patients with retinal degenerative diseases.

Study design

The study was intended to demonstrate the usefulness of ciliary epithelium derived neurospheres in restoration of normal vision. Cicero *et al* have analyzed morphological features of ciliary epithelium from adult C57BL/6 mice using transmission electron microscopy (TEM). Pigmented ciliary epithelium from adult C57BL/6 mice was observed to have melanosomes, basal and lateral membrane interdigitations and epithelial cell-cell junctions. These features were not found in retinal progenitors endorsing that adult CE cells and retinal progenitors don't share any morphological characteristics. Molecular profiling of CE revealed that specific genes such as Palm1, Rab27b (which are expressed in the fetal and adult pigmented ciliary epithelium) were expressed only in the pigmented ciliary epithelium but not in the retina. Also, Nrl and recoverin genes are specifically expressed in retina as analysed with real time PCR further delineates pigmented CE from retina. CE derived spheres were also examined for the presence of retinal stem and/or progenitor cells by RT-PCR (molecular), immunocytochemistry (cellular) and TEM (morphological) and then compared with adult CE, retinal progenitors and retinal progenitor derived spheres (from P0 retina). CE derived spheres contained melanosomes, cell to cell junctions and membrane interdigitations as that of adult pigmented CE cells which were not found with retinal progenitor cells or retinal neurospheres. Real time PCR analysis of CE derived spheres revealed that, they share most of their genes with that of adult pigmented CE, but not with the retina.

To rule out the possibility that sphere are originating from pigmented CE and not from RSCs or progenitor cells present in the CE, authors analyzed CE from $Chx10^{orj/orj}$ mutant mice. $Chx10^{orj/orj}$ mutant mice are known to have more number of CE

cells and are expected to form more number of spheres. TEM analysis of spheres derived from this mutant mouse showed that each cell was pigmented and these spheres were derived from pigmented CE cells rather that RSCs or retinal progenitor cells. To further confirm these results, authors labeled proliferating cells in the sphere with [^{3}H] – thymidine on day 3 for 1hr and then stained with toluidine blue. When these spheres were analyzed by TEM imaging, they showed the morphological characteristics such as melanosomes, membrane interdigitations and epithelial junctions as that of adult pigmented CE.

Although the data indicated that proliferating cells were found to be pigmented CE cells, however the authors have not excluded the possibility of a rare stem cell population. To analyze every cell in the CE derived neurosphere, they performed serial electron microscopic analysis and morphometric analysis of entire sphere. Out of 383 cells analyzed, all the cells showed pigmented CE cell features and none of the cell was non pigmented RSC or neural progenitor cell. *Nestin* expression was observed in spheres derived from pigmented CE under proliferation conditions or stem cell medium. These pigmented CE cells may ectopically up-regulate stem cell markers upon exposure to stem cell medium despite their differentiatiated state. To distinguish between these two possibilities, authors cultured the cells in the presence and absence of stem cell medium. During this period, at several time points (0,2,8 and 24hrs), cells were tested for nestin expression. Results showed that, exposure to stem cell medium were sufficient to up-regulate the nestin expression and these nestin expressing spheres contained pigment, membrane interdigitations and epithelial juctions similar to that of differentiated CE. It indicates that ectopic expression of nestin did not mark stem cell population in the CE or in the CE derived spheres. Previous studies showed that, there was robust transdifferentiation between CE, retina and RPE in various species. To test this hypothesis, authors plated CE derived spheres on laminin coated coverslips and maintained in serum containing media for 21 days. Under these differentiation conditions, each cell retained pigment and TEM analysis revealed that, the cells retained all morphological features of CE cells and none of the retinal neurons. They also performed RT PCR on cells kept for differentiation for every 2 days for 21 days. The genes that normally express in the rods (Nrl, Rhodopsin, Gnat1), bipolar cells (PKC α) and Muller glia (Carlbp) were not induced significantly as that of retina. But, genes like Palm1, Rab27b were increased in these cultures. Along with RT PCR, they have also tested the retinal markers by immunocytochemistry. Majority of cells were positive for cytokeratin, marker for pigmented epithelial cells. These results showed that, all the labeled cells retained the pigment and morphology of CE cells. Experiments with human CE derived spheres showed same results as that of murine CE derived spheres.

Finally, to determine the transdifferentiation capacity of CE derived spheres in new borns, cells from CE derived spheres were injected into the subretinal space of new born pups and eyes were analyzed after 21 days. They did not find any integration of these cells in the developing retina and injected cells were grouped together and formed basal lamina. These results have indicated that, CE derived spheres don't have the capacity to integrate into the retina. But, authors didn't transplant these cells in any retinal degenerative model (don't start a sentence with). All the results showed that, CE derived spheres do not share any of its profiles with that of retinal progenitors. As these CE derived spheres are pigmented, these cells don't appear to be a good source for transplantation in any retinal degenerative disease.

Implications

Characterization of CE stem cells by Cicero et al., yields information for future utility of these cells in treating retinal degenerative diseases. Though pigmented CE spheres are not good source for transplantation authors didn't mention how and why these cells possess unique proliferation capacity. Further research is required to achieve transdifferentiatation of the CE stem cells into pure retinal stem or progenitor cells which can be used for the treatment the retinal degenerative diseases.

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References

1. Pandey P, Pradhan S. Homocysteine: a possible modifiable risk factor in vascular dementia. Annals of Neurosciences 2006;13(1):12-18.
2. Perron M, Harris W A. Retinal stem cells in vertebrates. Bioessays. 2000;22(8):685-8.
3. Raymond P A, Barthel L K, Bernardos RL, *et al*. Molecular characterization of retinal stem cells and their niches in adult zebrafish. BMC Dev Biol. 2006;6:36.
4. Ahmad I, Tang L, Pham H. Identification of neural progenitors in the adult mammalian eye. Biochem Biophys Res Commun. 2000;270(2):517-21.
5. Tropepe V, Coles B L, Chiasson B J, *et al.* Retinal stem cells in the adult mammalian eye. Science. 2000;287(5460):2032-6.
6. Lord-Grignon J, Abdouh M, Bernier G. Identification of genes expressed in retinal progenitor/stem cell colonies isolated from the ocular ciliary body of adult mice. Gene Expr Patterns. 2006;6(8):992-9.
7. Gu P, Harwood L J, Zhang X, *et al.* Isolation of retinal progenitor and stem cells from the porcine eye. Mol Vis. 2007;13:1045-57.
8. Inoue Y, Yanagi Y, Tamaki Y, *et al.* Clonogenic analysis of ciliary epithelial derived retinal progenitor cells in rabbits. Exp Eye Res. 2005;81(4):437-45.
9. Jonas J B, Panda-Jonas S, Singh Hayreh S. Retinal progenitor cells in the posterior pars plana of rhesus monkeys. Br J Ophthalmol. 2004 Jun;88(6):836-7.
10. Xu H, Sta Iglesia D D, Kielczewski J L, *et al.* Characteristics of progenitor cells derived from adult ciliary body in mouse, rat, and human eyes. Invest Ophthalmol Vis Sci. 2007;48(4):1674-82.