Biology and therapeutic potential of adult retinal stem cells

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ABSTRACT • RÉSUMÉ

Retinal degeneration encompasses a constellation of common pathologies for which there is no regenerative treatment. Vision loss has a devastating impact on quality of life and activities of daily living. Pharmacologic treatments serve to stave off disease progression but do not represent a restorative approach. Cellular transplantation is considered to be a promising approach for future therapy for retinal degeneration. There are, however, significant barriers that must be overcome if cell transplantation is to become a clinical reality. In this review, we focus on the need for a cellular replacement therapy for retinal disease and the promise of stem cells as candidate cellular therapeutics. In particular, we discuss the origins of stem cells in the retina, the discovery and characterization of retinal stem cells isolated from adult humans, and their transplantation potential and clinical implications.

La dégénérescence de la rétine comporte une constellation de pathologies communes pour lesquelles il n'y a pas de traitement régénérateur. La perte de la vision a un effet dévastateur sur la qualité de vie et les activités quotidiennes. Les traitements pharmacologiques servent à prévenir la progression de la maladie mais ne présentent pas une approche restauratrice. La greffe cellulaire est considérée comme étant une approche prometteuse de thérapie éventuelle pour la dégénérescence de la rétine. Il y a cependant d'importantes barrières à franchir pour qu'elle devienne une réalité clinique. Dans cette revue, nous nous concentrons sur le besoin d'une thérapie de remplacement cellulaire pour la maladie de la rétine et la promesse des cellules souches comme candidates à la thérapie cellulaire. Nous discutons particulièrement des origines des cellules souches dans la rétine, de la découverte et la caractérisation des cellules souches de la rétine isolées des humains adultes et des possibilités et implications cliniques de leur greffe.

THE CLINICAL IMPETUS FOR RETINAL CELLULAR REPLACEMENT THERAPY

Diseases of the retina lead to permanent loss of visual function for which there is no definitive treatment. Retinal degenerative diseases affect the entire age spectrum. Age-related macular degeneration (AMD) is the leading cause of irreversible blindness and moderate visual impairment in developed nations: it affects more than 2 million Canadians over the age of 50 years.[1] Diabetic retinopathy is the principal cause of blindness in middle-aged working adults.[2] Retinitis pigmentosa (RP) affects predominantly the pediatric and young adult population, and is the leading cause of blindness associated with inherited retinal degeneration.[3] What all of these pathologies have in common is irreversible photoreceptor death or loss of function. Driving forward the search for cures is the fact that as our population ages in the coming decades, it is expected that the rates of blindness due to retinal degeneration will rise.[4,5]

Current therapies for retinal diseases have focused on pharmacological treatments. For example, there have been recent advances in the treatment of the neovascular (wet) form of AMD with antivascular endothelial growth factor therapies.[6,7] Experimental treatments of diabetic retinopathy focus on bioactive molecules such as inhibitors of advanced glycoslyation end products and antioxidants.[8] These therapies show promise in limiting the pathophysiologic advancement of the disease; they do not represent a restorative approach to vision loss. Cellular transplantation is an alternative strategy. The inner retinal microarchitecture in both AMD and RP is relatively intact after photoreceptor degeneration, and 1 approach would be to repopulate the missing photoreceptor cells. Various types of tissue have been allografted in the treatment of retinal disease: fetal retinal pigmented epithelium (RPE) cells in patients with AMD[9,10] and neural retinal cells in patients with RP.[11] While graft survival is observed, the improvement in visual acuity has been disappointing.[12]

Recent studies suggest that stem cell transplantation shows promise for reconstituting damaged cell populations in the retina.[13,14] Stem cells are the most versatile cells in a living organism. They are defined by their self-renewal and multipotentiality: their ability to generate specialized progeny of various cell lineages. As a cell source for future cell replacement therapy, stem cells are among the most promising for the treatment of injured, diseased, or aging

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tissues. Embryonic stem cells, adult stem cells, and induced pluripotent stem cells are the 3 main classes of stem cell and are derived from different sources. All have their own advantages and hurdles that must be cleared before they can be utilized for therapy.[15] In this review, we will consider the various types of potential stem cell sources before turning our attention to the history of stem cells in the retina. We will cover the discovery of the adult mammalian retinal stem cell, its characterization, transplantation potential, and possible future clinical application.

Stem cells: what they are and where they come from

Embryonic stem cells are pluripotent cells isolated from the inner cell mass of preimplantation blastocysts. They can give rise to differentiated cell types of all 3 germ layers of the organism and rapidly expand in culture. However, they are inefficient at differentiation along a particular lineage, and safety is an important concern in their clinical application. Their proliferative ability may lead to tumour and mass formation.[16] Predifferentiation of these cells is 1 means to diminish the implied risk of transplanting a pluripotent population directly.[17] However, in the absence of directed differentiation and a means by which to purify a cell population, impure populations of differentiated cells can arise.[18]

Induced pluripotent stem cells, which have all of the properties of bona fide embryonic stem cells, can be generated by introducing 4 genes (Oct4, Sox2, Klf4, c-Myc) into somatic cells. These somatic cell types include adult fibroblasts, among other tissues such as liver and stomach.[19–21] Recently, programming with as few as 2[22] or 1[23] of these factors has been reported. Transgene-free methods of induced pluripotent stem cell generation are also being explored.[24,25] Induced pluripotent stem cells benefit from being immune compatible as a result of their derivation from adult somatic cells of the same patient, and they overcome the ethical issues faced by the use of embryonic stem cells. However, the absence of a method to direct these cells into a single lineage reproducibly and to avoid the risk of tumour formation has restricted their use in humans.[26,27] Also, the current methods of somatic cell reprogramming are still slow and inefficient.[26] Investigators have developed protocols for the generation of retinal cell types, such as photoreceptors, from human embryonic stem cells[28–31] and induced pluripotent stem cells.[32] However, without going through a definitive retinal stem cell type, these methods cannot be certain to exclude nonretinal cell types.

While multipotent adult stem cells lack the potency to generate any cell type in the body, they are not considered tumourigenic. They are also primed for the generation of progeny that differentiate into the specific cell types of the tissues in which they are resident and with high efficiency. Amplification of populations of adult stem cells using in vitro culture to yield clinically useful numbers is the major challenge, which will require an understanding of the biology governing the maintenance of the stem cell niche in vivo.[33,34]

History of the stem cells in the eye: evidence for multipotential stem cells and progenitors in the vertebrate retina

The interest in stem cells and their role in the retina during development and adult life spans just over 2 decades of research. The experiments leading to the discovery of the adult mammalian retinal stem cell began with investigations of retinal development from multipotential retinal progenitors.

In the late-1980s, using retrovirus-mediated gene transfer and lineage marking, it was discovered that a common progenitor exists in the developing mammalian retina for neurons and glia.[35] Similar multipotent retinal progenitor cells (RPCs) can give rise to all of the major cell types of the adult nonmammalian vertebrate (xenopus) retina.[36,37] Together, these early studies showed that RPCs can give rise to heterogeneous clones, but it was still unclear whether multipotency was a common feature of all retinal progenitors or whether this potency became more restricted with developmental age.

In the mid 1990s, the differentiation capacity of these RPCs was better understood. A model was developed in which progenitors undergo a series of state changes defined by the competence to respond to environmental cues to

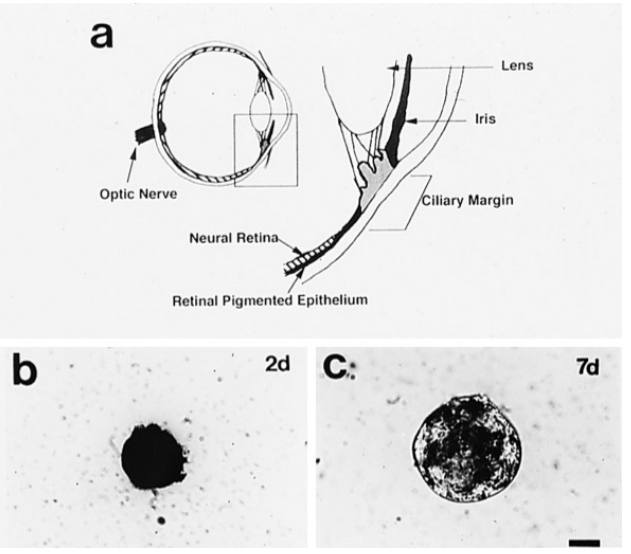

Fig. 1—Sagittal section of the ciliary margin of the adult human eye (A). The boxed area is magnified on the right. The ciliary margin is made up of pigmented cells (dark inner line) overlying the smooth muscle of the ciliary body (grey) facing the lens. A clonally derived sphere of cells derived from the pigmented ciliary margin (B). All cells in the sphere are pigmented and derived from a single pigmented cell. After 7 days, cells proliferate to form large colonies (100 μm+) containing pigmented and nonpigmented cells (C). Reprinted with permission.[60]

produce 1 or a few particular cell types.[38] Progenitors lose competence for cell types produced earlier in development, as suggested by experiments carried out in vitro[39,40] and by transplantation of RPCs between developmental stages in vivo.[38,41,42] Recently, isolation and experimental transplantation of RPCs into murine and porcine retina have been described.[43,44] The integrated cells express markers of mature photoreceptors, such as rhodopsin. However, RPC cells must be isolated from the neural retina of the developing eye, and this again raises ethical issues surrounding the transplantation of fetal tissue.

Concurrently, the adult regenerative potential of the vertebrate retina was being evaluated in various animal models. In cold-blooded vertebrates (e.g., teleosts/zebrafish) the retina continues to grow throughout life and in response to injury in the adult. This occurs by the addition of new neurons at the rim of the retina from a germinal zone at the ciliary margin.[45] Müller glial cells can dedifferentiate in response to injury and produce neuronal progenitors in fish.[46] Also, transdifferentiation of the RPE into neural retina has been demonstrated in a number of amphibians, as well as in embryonic chick and rat.[47–50] A proliferating marginal zone of retinal progenitors in postnatal chickens had been identified containing cells that share similarities with RPCs and the proliferative cells of the cold-blooded vertebrate retinal margin.[51] However, it had been generally assumed that the adult mammalian eye was devoid of retinal stem cells.[52–59]

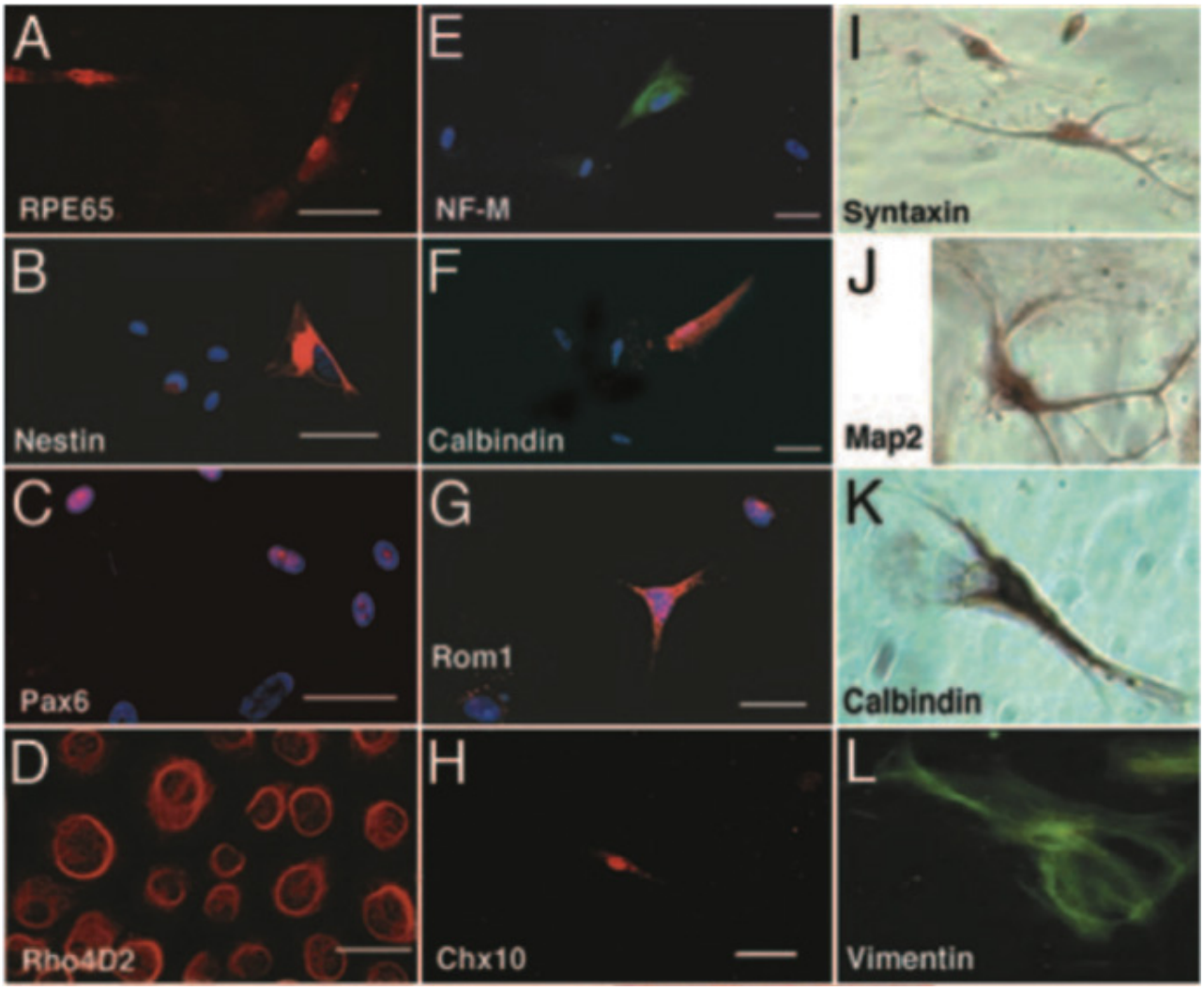

Fig. 2—Multipotency of retinal stem cells is demonstrated by culture on laminin-coated plates and exposure to differentiation conditions (1% fetal bovine serum + basic fibroblast growth factor). Nuclei are stained blue with Hoechst in B, C, and E–G. The differentiated progeny include RPE cells (RPE65+) (A), undifferentiated cells (nestin+) (B), retinal progenitors (nuclear)/amacrine (cytoplasmic) (Pax6+) (C), photoreceptors (Rho4D2+) (D), retinal ganglion cells (neurofilament-M+) (E), horizontal cells (calbindin+) (F), photoreceptor cells (Rom1+) (G), bipolar cells (Chx10+) (H), neuronal markers (syntaxin+, MAP2+, calbindin+) (I–K), and glial lineage (vimentin+) (L). Scale bars: 20 μm. Reprinted with permission.[61] (RPE, retinal pigmented epithelium.)

Discovery of the adult mammalian retinal stem cell

In 2000, Tropepe and colleagues[60] reported the isolation of a stem cell in the adult mouse eye that represented a promising cell type for retinal regeneration. These adult retinal stem cells (RSCs) are localized in the pigmented ciliary margin and not in the central or peripheral RPE (Fig. 1). This indicated that the cells might be homologous to the germinal zone of lower vertebrates. The analogy has been made between the pigmented epithelium of the ciliary body and the ciliary marginal zone of lower vertebrates, in which the adult germinal zone lies. While the ciliary marginal zone is an undifferentiated neuroepithelium,[37] the pigmented epithelium of the ciliary body is a mature, differentiated epithelial monolayer. It is physically distinct from the retina and lies anterior to the ora serrata. In culture, RSCs proliferate to form clonal spheres of stem cells and progenitors. The ability for self-renewal was demonstrated through the production of multiple secondary spheres from the passaging of a single sphere.[60] Most excitingly, these progeny are multipotential and can differentiate into all cell types of the retina, including photoreceptors, bipolar cells, RPE cells, and Müller glia. This ability sparked interest in their potential for the treatment of human retinal diseases.

Four years later, the isolation of human RSCs was reported,[61] from donor eyes ranging in age from the early postnatal period to the seventh decade. These adult stem cells were also characterized in terms of their self-renewal and multipotency (Fig. 2). To assess their potential as a cellular therapeutic, the undifferentiated RSC progeny were transplanted into postnatal day 1 mice. In mice, photoreceptor genesis peaks at postnatal day 1.[38,62] These cells were able to survive, migrate, integrate, and differentiate into the neural retina. In particular, a large proportion of them integrated into the outer nuclear layer and took up a photoreceptor phenotype (Fig. 3). Transplantation of human RSCs into embryonic chick eyes demonstrated that RSC progeny could also respond to developmental cues to form ganglion and horizontal cells—cell types that form earlier in retinogenesis.[38]

Since this time, significant emphasis has been placed on understanding the basic stem cell biology of adult RSCs, in particular understanding their position in the retinal lineage. A better understanding of the factors that maintain RSC multipotency and self-renewal could open the door to enhancing the efficiency of their derivation and (or) differentiation. For example, it has been shown that RSCs are enriched for Pax6, a master control gene for establishment of the retinal field from forebrain neuroectoderm. This transcription factor is essential for the proliferation and expansion of RSCs in vitro.[63] The undifferentiated RSC progeny also express markers of undifferentiated retinal cells such as Chx10[60,61,64] and the neural stem/progenitor marker Nestin.[60,61] Multipotent cells have been reported in the adult mammalian iris pigmented epithelium.[61,65] However, in humans only the posterior iris contains colony-forming cells that proliferate to form primary spheres, which cannot be passaged to secondary spheres.[61] This suggests that sphere-forming cells derived from the posterior iris may represent a progenitor population with some limited proliferative ability. Developmentally, the posterior iris arises from a neuroectoderm similar to that of the ciliary body and retina, whereas the anterior iris is derived from the neural crest.[66] This may explain the isolation of a retinal progenitor population from the posterior iris only.

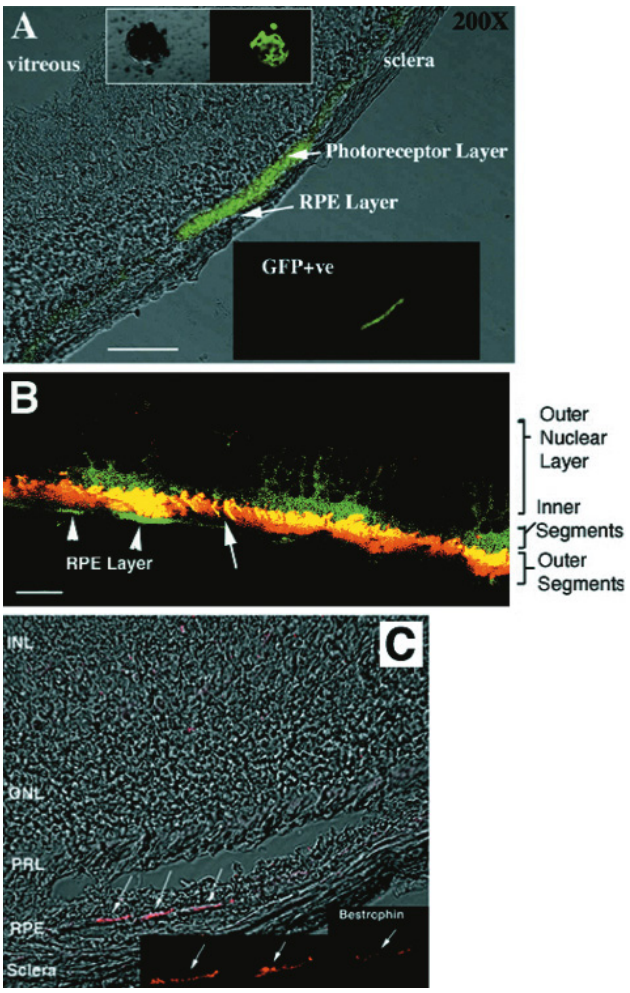

Fig. 3—Transplantation of RSC progeny into postnatal day 1 NOD/SCID mouse eye. Green fluorescent protein (eGFP+) human RSCs and progeny integrate into the neural retina and RPE (A). Scale bar: 250 μm. Inset: the human RSC sphere (250 μm diameter) derived from an eGFP labeled RSC in phase-contrast and under green fluorescence. eGFP+ RSC progeny can produce photoreceptors that integrate into the outer nuclear layer of the neural retina (Rom1+ outer segments stained red appear yellow as a result of colocalization with eGFP) (B). Arrowheads indicate eGFP RSC progeny integrated into the RPE. Scale bar: 50 μm. Transplanted human RSCs can integrate into the RPE layer (bestrophin+ cells) (C). Reprinted with permission.[61] (RSC, retinal stem cell; NOD/SCID, nonobese diabetic/severe combined immunodeficiency; RPE, retinal pigmented epithelium; INL, inner nuclear layer; ONL, outer nuclear layer; PRL, photoreceptor layer.)

Recently, it has been proposed that the RSC population does not constitute a true stem cell population. Rather, the observed multipotency is claimed to be the result of transdifferentiation of a differentiated pigmented ciliary epithelial cell by induction of stem/progenitor markers in response to growth factors in the culture media.[67] This group's arguments against stemness include the following: (*i*) the cells isolated from the ciliary epithelium include all of the pigmented cells here and (*ii*) poor retinal differentiation has been observed, with unconvincing photoreceptor differentiation in particular.[67] However, there is strong evidence against these propositions to suggest that the RSC population represents, in fact, true stem cells and can give rise to multiple retinal lineages. The original reports of the RSC population specifically reference that the proliferative and multipotent cell derived from the ciliary epithelium is pigmented,[60,64] and the specific rare population of RSCs from the ciliary margin have high Pax6 expression.[63] Rare stem cells can be prospectively isolated on this basis. Transdifferentiation implies differentiation of a single cell without proliferative divisions, which is not the case with RSCs derived from ciliary epithelium. Furthermore, the multipotency of the RSC and its ability to differentiate into various retinal cell types, including photoreceptors, have been corroborated by a number of independent reports.[60,61,64,68–71]

TRANSPLANTATION POTENTIAL OF RSCs: THE CLINICAL FRONTIER

Research is progressing to understand the factors maintaining the stem cell niche in vivo. However, it is unlikely

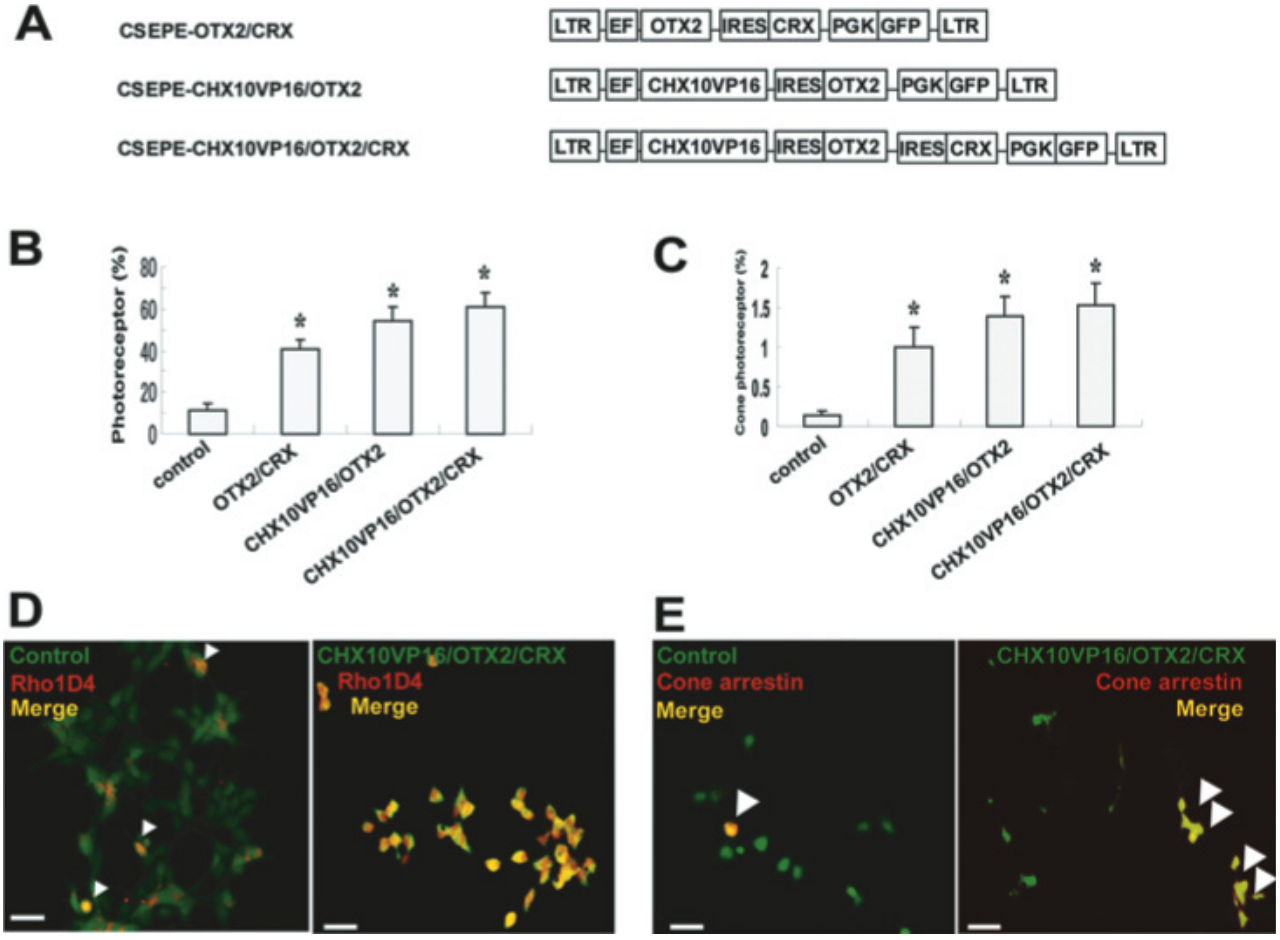

Fig. 4—Transduction of Otx2[89] and Crx[90] combined with reversal of Chx10[91–93] to an activating form, using Chx10VP16, produces the most potent induction of photoreceptor differentiation from human RSCs. Self-inactivating lentiviral vector CSEIE containing internal ribosomal entry site (IRES) sequences followed by enhanced green fluorescent protein (eGFP) (A). Constructs for the expression of Otx2, Crx, and Chx10VP16 were cloned into this vector, which directs the expression of the cloned genes together with eGFP from the internal promoter (EF1α) to be tested for their photoreceptor induction potential. The 3 tested transfections included the combinations Otx2/Crx, Chx10VP16/Otx2, and Chx10VP16/Otx2/Crx10. Photoreceptor differentiation was significantly promoted by coexpression of these 3 constructs compared with controls for rods (B) and cones (C) (ANOVA and Dunnett's multiple comparison test, $*p < 0.05$). Rho1D4+ rods (D) or human cone arrestin+ cells (E) coexpress GFP from the control (left panel) or Chx10VP16/Otx2/Crx-expression vector (right panel) as illustrated by yellow fluorescent overlap. Many more rods and cones were formed during differentiation using Chx10VP16/Otx2/Crx-transduction than control transduction (eGFP+ cells). Reprinted with permission.[68] (RSC, retinal stem cells; ANOVA, analysis of variance.)

that therapeutics aimed at stimulation of the proliferation and differentiation of endogenous RSCs will be successful. Migration of RSC progeny from the ciliary margin to the central fovea, the primary site of photoreceptor degeneration in diseases such as AMD, would need to occur over a significant distance. It is also unclear what signals, if any, could be used to enhance this migratory process. At present, a transplantation model of therapy would appear to be the most clinically relevant. For RSCs to have therapeutic potential their ability to integrate and restore vision in animal models of retinal disease is essential. Experiments involving the transplantation of RSCs into retinas devoid of photoreceptors or into partially degenerated retinas have shown that the adult retina, whether partially degenerated or already degenerated, cannot itself provide the signals to induce differentiation of RSCs into photoreceptors.[72] There are a number of potential explanations, including insufficient contact between graft and host cells as a result of poor integration of RSCs into the outer nuclear layer, observed in subretinal transplantation.[72–74] These findings suggest that a promising approach could be to push differentiating RSCs, before transplantation, towards a neuronal lineage. Reports of enhanced integration potential of progenitors primed to become mature photoreceptors support this hypothesis.[75]

A major limitation in using the progeny of RSCs to replace photoreceptors is that these cells are only a minority of differentiated RSCs in vitro. To address this problem, the expression of 3 genes known to influence photoreceptor development were manipulated using a lentiviral-mediated gene delivery system (Fig. 4A).[68] The efficiency of photoreceptor induction was greatly increased, as demonstrated with in vitro differentiation and transplantation into adult mouse eyes (Fig. 4B–E). Furthermore, human RSC progeny transfected with the 3 genes could adopt photoreceptor fates more efficiently after transplantation into postnatal day 1 mice, and contributed to functional recovery when transplanted into transducin-mutant mice.[76] In transducin-mutant mice, rod photoreceptors are present but do not function. Functional recovery was demonstrated with electrophysiological (electroretinography, ERG) and behavioural assays 3 months after transplantation (Fig. 5). At high flash intensities, ERG b-wave (bipolar) responses, reflecting cone photoreceptor activation, were not different between control and transfected groups. However, under low-intensity light, the ERG b-wave responses repeated measurements demonstrated that cells treated with the 3 genes show a higher response, corresponding to the integrated cells observed by histology. B-wave responses should be the best reflection of integrated donor human photoreceptors that have made functional connections to host bipolar cells.[77] In a virtual optomotor task[78] all transplanted eyes showed better spatial frequency resolution than nontransplanted eyes, and transfected cells showed better spatial vision than control transplanted eyes (Fig. 5). These results are strong evidence in support of the potential of adult RSCs to provide functional recovery after transplantation.

As a transplant model, the developing mouse host eye provides a permissive environment for donor cells integrate and respond to endogenous cues directing retinogenesis.

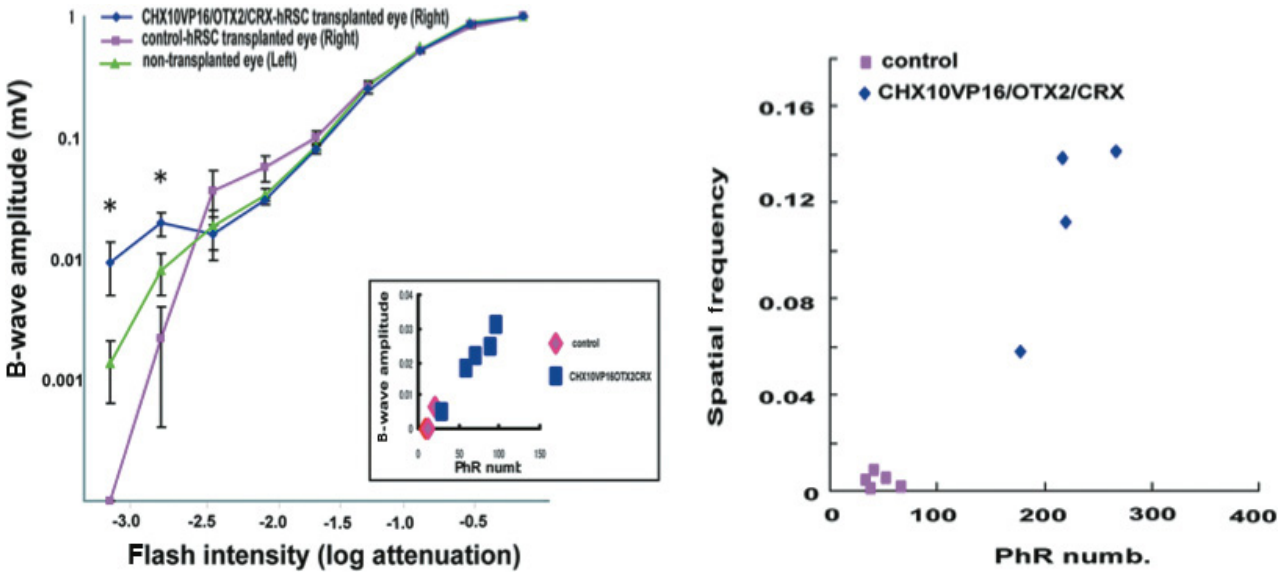

Fig. 5—Function of transplanted human RSC progeny in vivo. At the lowest flash intensities (left panel), the transducin-mutant group receiving Chx10VP16/Otx2/Crx-transfected human RSCs shows a higher response than the nontransplanted or eGFP-only vector treated groups (ANOVA and Dunnett's multiple comparison test, $*p < 0.05$). Inset shows a significant correlation in maximal b-wave response and surviving human photoreceptor cell numbers counted in survival/integration studies. The differences in spatial frequency data (right panel) were estimated and represent intra-animal controls between the transplanted (right eye) and nontransplanted (left eye) in each individual mouse. Chx10VP16/Otx2/Crx-treated cells showed better spatial vision than control transplanted eyes. Reprinted with permission.[68] (RSC, retinal stem cells; ANOVA, analysis of variance.)

Furthermore, a mature glial limitans membrane is not present, which would prevent transplanted cells from migrating into the neural retina in adult intravitreal cellular transplantation.[79] In the future application of stem cells or their progeny for the treatment of retinal pathology in adults, the mature glial limitans must be bypassed: the target for adult cellular replacement therapy is subretinal. Barriers to adult subretinal transplantation include cellular

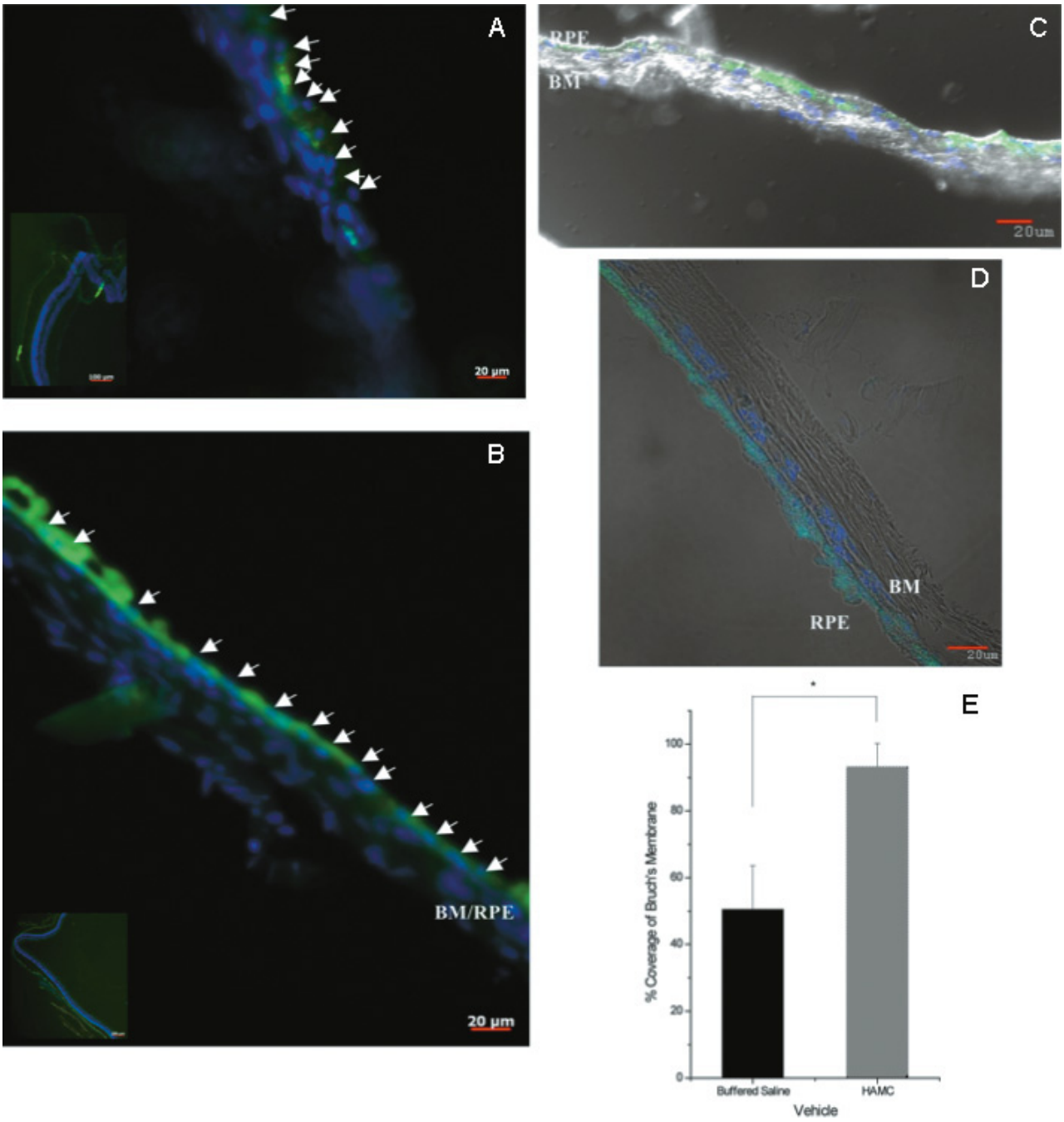

Fig. 6—Subretinal transplantation of GFP+ RSC progeny in the vehicle, a physical blend of hyaluronan (HA) and methylcellulose (MC)–HAMC–assayed at 4 weeks' post-transplantation. Transplantation in saline shows noncontiguous cellular integration and localized cellular aggregates (inset) atop Bruch's membrane (BM) (A), suggestive of aggregation before or after transplantation. Transplantation in HAMC shows contiguous areas of RPE integration over large areas of retina (inset) (B), suggesting that HAMC maintains cellular distribution during injection and prevents aggregation before or after transplantation. Arrowheads indicate location of individual nuclei of transplanted cells (Hoechst nuclear stain, blue). Confocal images of cuboidal RPE cells sitting atop Bruch's membrane after injection in saline (C) or HAMC (D) (merged with contrast to show cytoplasmic architecture). Note noncontiguous distribution in HAMC versus buffered saline vehicle. Integration along Bruch's membrane shows significantly greater coverage by GFP+ cells delivered in HAMC versus buffered saline over areas of observed integration (n = 3 eyes each) (E). Reprinted with permission.[74] (GFP, green fluorescent protein; RSC, retinal stem cell; RPE, retinal pigmented epithelium.)

survival and integration into host tissue. It has been well documented that cell death, leakage, and migration from the transplantation site occur when retinal progenitor cells are delivered as a single-cell suspension in saline.[80] To overcome the survival and integration barriers in the adult, interdisciplinary studies, such as the combination of regular tissue culture with tissue engineering, are being used. To date, these have included delivery of retinal progenitor cells, isolated during development, on solid biomaterial scaffolds.[81–84] While important advances have been made, these solid scaffolds do not match the modulus of the retina and lack the flexibility required for subretinal delivery.[84]

Recently, a minimally invasive, injectable, in situ, biodegradable cellular delivery matrix vehicle has been developed for the transplantation of adult RSCs to the subretinal space of adult mice.[74] The vehicle allows for normal RSC survival and proliferation in vitro and exhibits benefits in overcoming barriers to cell integration in vivo compared with saline controls (Fig. 6). Tissue analysis at 4 weeks after transplantation revealed that RSCs delivered subretinally in saline resulted in noncontinuous integration into the RPE, whereas RSCs in the polymer vehicle integrated with the RPE and formed continuous banding atop Bruch's membrane. Delivery in the polymer vehicle greatly increased coverage of Bruch's membrane over the area of subretinal injection (Fig. 6E). This cell delivery strategy may be useful for the treatment of widespread and (or) advanced maculopathy, in which large areas of RPE are destroyed.[85] The choroidal neovascularization that is a hallmark of wet AMD is marked by widespread RPE disruption and disturbance of homeostatic mechanisms of photoreceptor outer segment phagocytosis.[86–88]

Conclusions

The field of adult RSC research has much to offer those interested in the science and treatment of retinal degenerative diseases. The response of transplanted RSCs to various forms of retinal injury or degeneration gives a method to investigate the pathologic mechanisms at work in the diseased microenvironment. From a clinical perspective, RSC transplantation has shown that the actively degenerating or dystrophic adult retina can be repopulated with donor-derived retinal cell types. These new cells can survive and exhibit morphological and functional integration with host neurocircuitry. While much remains to be improved in terms of integration efficiency and improved visual acuity, the field presents an exciting new strategy for retinal disease management and the hope that effective regenerative treatments are not far in the future.

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