Stem Cell Biology and Regenerative Medicine

Stephen H. Tsang *Editor*

Stem Cell Biology and Regenerative Medicine in Ophthalmology

Humana Press

Stem Cell Biology and Regenerative Medicine

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Humana Press

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ISBN 978-1-4614-5492-2 ISBN 978-1-4614-5493-9 (eBook)
DOI 10.1007/978-1-4614-5493-9
Springer New York Heidelberg Dordrecht London

Library of Congress Control Number: 2012951349

Printed on acid-free paper

Humana Press is a brand of Springer
Springer is part of Springer Science+Business Media (www.springer.com)

Preface

In the last few decades, stem cell research has developed groundbreaking technologies to both study and treat diseases. This research has proven fruitful for the field of ophthalmology, especially in recent years. With its relative immune privilege, the eye has proven an ideal testing ground for stem cell therapies.

This book describes just a few of these developing treatments. The authors of this book describe a wide range of possible applications, from oculofacial plastic surgery to the restoration of sight lost by degenerative disorders and glaucoma, to cancer research. Indeed, stem cell research seems to have reached a critical mass in ophthalmology. As recently as 2011, the FDA approved trials for stem cell-based treatments for macular degeneration; other clinical trials may follow, as discussed in the last chapter of this book.

These changes have not happened overnight. From a scientific standpoint, several discoveries have made stem cells a viable treatment source for humans. In 1981, when embryonic stem cells were first synthesized in the laboratory, it became possible to imagine generating graft tissues or animal models to test drugs from stem cells. Fifteen years later, the Yamanaka research group discovered that mouse skin samples could be reprogrammed through gene therapy into induced pluripotent cells. Both ES and iPS cells are pluripotent, or reprogrammable. Moreover, iPS cells are autologous, meaning they are derived from the subject's own tissue. By modifying cell culture media or performing gene therapy, researchers have been able to generate many types of tissues using ES cells and iPS cells.

Autologous tissues can also be generated using the progenitor cells which exist naturally inside the body. Unlike pluripotent stem cells, progenitor cells can differentiate into a limited number of tissues. These "local" cells can be adapted to replace and repair diseased tissue. Promising progenitor cells include: adipose tissue stem cells, ciliary stem cells, mesenchymial stem cells, corneal stem cells, and lens stem cells.

A significant area of stem cells research has been the retinal degenerative disorders. These conditions all involve degeneration of the retinal pigment epithelium, a tissue that sustains living photoreceptors. Researchers have hoped to restore this tissue with differentiated stem cells. To date, several studies have found visual

rescue in mice treated with stem cell-derived RPE and photoreceptors. Intricate new surgical techniques have had to be developed to perform these transplant procedures.

Transplant surgeries can be used to replace many kinds of damaged tissue. Recently, adipose tissue-derived stem cells have attracted interest as source of tissue for oculofacial surgeries such as facial reconstruction, wound healing, and skin rejuvenation. The ease of gathering these autologous stem cells makes them particularly advantageous for plastic surgeries.

Stem cell-derived tissues such as lens and corneal tissue may be suitable for transplant. Media outlets have already begun reporting on the potential that severe corneal epithelial diseases may be treatable with corneal stem cells. Successful efforts have also been made to generate lens progenitor cells and lentoid bodies from ES stem cells.

Research on mesenchymial (or, marrow) stem cells may allow treating vascular disorders of the eye. Recent findings suggest that these can be transplanted into the eye to improve angiogenesis. Bone marrow cells may have potential for treating ischemic retinal diseases, and perhaps even some non-ischemic retinal diseases.

The treatment of glaucoma may involve special challenges. It has recently been discovered that transplantation of stem cells into the retina can potentially replace damaged neurons, or provide neurotrophic factors to surviving neurons. These may be useful for treating glaucoma, providing that neurons are able to integrate. A number of signaling and transcription factors are currently being studied to this end.

Gene therapy has continued to evolve alongside stem cell therapy. New gene therapy techniques using gene addition, or enhancing gene replacement, have improved the efficiency of directly treating disease-causing genes. These gene therapy methods minimize the risk of mutagenesis and may be used along with stem cells to replace diseased patient cells with new disease-free cells.

Finally, stem cell research has improved our understanding of the pathogenesis of eye diseases. The mechanisms leading to various types of cancer are still unknown. This book contains a discussion of the evidence that cancer stem cells can lead to uveal melanoma.

Stem cell research never stops changing and growing. There may come a time when the research discoveries of today alter the landscape of ophthalmologic practice. The last chapter of this book describes the types of safety trials that may be used to assess stem cell-based treatments' viability. In the meantime, these cells of great potential continue to offer challenges to researchers and hope to patients with serious eye pathologies.

New York, NY, USA S. H. Tsang

Acknowledgments

This book would not have been possible without the generous support of P30EY019007 (Core Support for Vision Research), EY018213, and EY019861 from the National Eye Institute, National Institutes of Health, Bethesda, MD; Foundation Fighting Blindness, Owings Mills, MD; and unrestricted funds from Research to Prevent Blindness Inc, New York, NY. SHT is a Fellow of the Burroughs-Wellcome Program in Biomedical Sciences and has been supported by the Bernard Becker Association of University Professors in Ophthalmology Research to Prevent Blindness Award, the Foundation Fighting Blindness, the Joan and Michael Schneeweiss Research Fund, the Dennis W. Jahnigen Award of the American Geriatrics Society, the Joel Hoffman Scholarship, the Crowley Research Fund, and the Barbara & Donald Jonas Family Funds.

Contents

Abbreviations

3D	Three dimensional
AAV	Adeno-associated virus
ACAID	Anterior chamber-associated immune deviation
adRP	Autosomal dominant retinitis pigmentosa
ADSC	Adipose-derived stem cells
AMD	Age-related macular degeneration
AMED	Amniotic membrane matrix-based ES cell differentiation
AO	Adaptive optics
arRP	Autosomal recessive retinitis pigmentosa
ARVO	Association for Research in Vision and Ophthalmology
AT region	Adenine-plus-thymine region
BAC	Bacterial artificial chromosome
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
bHLH	Basic helix-loop-helix
BMP	bone morphogenetic protein
BMSC	Bone marrow stem cells
CAL	Cell-assisted lipotransfer
CAL	Conjunctival allograft
CAU	Conjunctival autograft
c-CLAL	Cadaveric conjunctival limbal allograft
CE	European Conformity
CEC	Corneal endothelial cell
CESC	Corneal endothelial stem cell
CLAU	Conjunctival limbal autograft
CMZ	Ciliary marginal zone
CNTF	Ciliary neurotrophic factor
CNV	Choroidal neovascularization
CoDA	Context-dependent assembly
CRX	Cone–rod homeobox
CTFR	Cystic fibrosis transmembrane conductance regulator

DNA	Deoxyribonucleic acid
EGFP	Enhanced green fluorescent protein
EPC	Endothelial progenitor cell
ERG	Electroretinogram
ES	Embryonic stem
ESC	Embryonic stem cell
FA	Fluorescein angiography
FAF	Fundus autofluorescence
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
fMRI	Functional magnetic resonance imaging
GCL	Ganglion cell layer
GFAP	Glial fibrillary acidic protein
GUCY2D	Retinal guanylate cyclase 2D
HCS	Hematopoietic stem cells
HES	Hairy enhancer of split
hESC	Human embryonic stem cell (see ES)
HIV	Human immunodeficiency virus
ICG	Indocyanine green
ILM	Inner limiting membrane
iPS	Induced pluripotent stem
iPSC	Induced pluripotent stem cell
ITR	Inverted terminal repeats
KLAL	Keratolimbal allograft
LCA	Leber's congenital amaurosis
LESC	Limbal epithelial stem cell
lr-CLAL	Living related conjunctival limbal allograft
LSCD	Limbal stem cell deficiency
LTR	Long terminal repeats
MEN	Multiple endocrine deficiency
mERG	Multifocal electroretinogram
mfERG	Multifocal electroretinogram (interchangeable)
MMP 1	Matrix metalloprotease 1
MS	Melanomasphere
MSC	Mesenchymal stem cell
MSFE	Melanosphere forming efficiency
NICD	Notch intracellular domain
NRL	Neural retina-specific leucine zipper
NSC	Neural stem cell
OCP	Ocular cicatricial pemphigoid
OCT	Optical coherence tomography
OPEN	Oligomerized pool engineering
OSD	Ocular surface disease
PAX-6	Paired boxed protein-6

PCP	Planar cell polarity
PCR	Polymerase chain reaction
PDM	Placental decellular matrix
PET	Positron emission tomography
PRO	Patient reported outcomes
rAAV vector	Recombinant adeno-associated virus vector
RCS	Royal College of Surgeons
REST	RE-1 silencing transcription factor
RGC	Retinal ganglion cell
RHO	Rhodopsin
RNA	Ribonucleic acid
RP	Retinitis pigmentosa
RPC	Retinal progenitor cell
RPE	Retinal pigment epithelium
RPGRL	Retinitis pigmentosa GTPase regulator
RVD	Repeat variable di-residue
SDIA	Stromal cell-derived inducing activity
SFEB/DLFA	Dkk-1, Lefty-A, FCS, and Activin cells
siRNA	Small interfering RNA
SJS	Steven–Johnson Syndrome
ssAAV vector	Single-strand adeno-associated virus vector
SVF	Stromal vascular fraction
TAC	Transient amplifying cell
TAL	Transcription activator-like
TALEN	Transcription activator-like effector nucleases
USH2A	Usher syndrome 2A
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VEP	Visually evoked potential
xlRP	X-linked retinitis pigmentosa
ZFN	Zinc finger nuclease

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Chapter 1
The Eye as a Target Organ for Stem Cell Therapy

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Abstract Retinal degenerations are a heterogeneous group of disorders that are characterized by progressive cellular dysfunction, cellular disarray, and eventually cell death. Early in the course of disease therapeutic intervention consists of pharmaceutical treatment to prevent cell death or gene therapy to correct the underlying mutation. Due to the nature of pathologies involving these disorders, particularly in late stage of disease, cell replacement therapy or electric stimulation of remaining cells by artificial retinal prosthesis is the only viable option. Stem cell therapies for retinal degenerative diseases such as age-related macular degeneration (AMD) and retinitis pigmentosa (RP) are a promising therapeutic option and will require replacement of lost photoreceptor cells and retinal pigment epithelium (RPE). Current clinical trials are underway to evaluate the potential of stem cell therapy in humans. The use of induced pluripotent stem (iPS) cells hold great promise as a potential reservoir of cells for the treatment of retinal disorders as well as a clinical tool to help understand disease pathology. Advances in stem cell technology will translate these therapies into viable clinical options for the treatment of retinal degenerative diseases and other disorders.

Introduction

Retinal degenerations are a heterogeneous group of disorders that are characterized by progressive cellular dysfunction, cellular disarray, and eventually cell death. Numerous classification systems exist for these disorders, but no one classification

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S.H. Tsang (ed.), *Stem Cell Biology and Regenerative Medicine in Ophthalmology*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-5493-9_1,

system captures the complexity of the disease processes, the diversity of their pathology, and the common themes in treatment that underlie these diseases. Many current classifications distinguish between macular diseases and peripheral retinal degenerations, but this classification system does not represent the complexity of the disease process in a complete fashion. Prior to the discovery of gene mutations that increase the risk profile for age-related macular degeneration (AMD), retinal degenerations were often classified as either hereditary or nonhereditary diseases, but the simplicity of this classification has been called into question based on the observation that certain alleles increased the risk of AMD [1–4]. Thus, for the purpose of this discussion, retinal degenerations will be classified by whether they are Mendelian disorders (e.g., most if not all forms of retinitis pigmentosa (RP), Leber's congenital amaurosis, and Best's disease) or non-Mendelian retinal disorders, including AMD.

Because of the complexity of the disease processes, it is possible to dedicate an entire chapter of this book to each disease and still not cover all the details of each condition. However, regardless of the cause of the retinal disorder, it is important to recognize that severe vision loss is typically associated with cellular dysfunction or death. Early in the course of many diseases there is cell dysfunction without cell death. In these early stages, gene therapy, pharmacological treatment to manipulate the cell death pathway, and/or treatment with locally administered growth factors, such as ciliary neurotrophic growth factor, may all prove to be useful. However, late stages of retinal disease, which are usually accompanied by severe vision loss, will require a different approach. For example, in advanced stages of many forms of RP, severe vision loss is due to death of photoreceptors, loss of the native retinal pigment epithelium (RPE) monolayer on Bruch's membrane, migration of pigmented cells into the retina, and transsynaptic degeneration leading to inner retinal disturbance. In advanced geographic atrophy in AMD, there is loss of RPE and photoreceptors and secondary atrophy of the choriocapillaris. Reversal of vision loss in these late stages of disease, after cell loss has occurred, will likely require cell therapy with transplantation of photoreceptors, RPE and/or choriocapillaris cells; or direct electrical stimulation of the inner neural retina with multi-electrode arrays.

In this review we will discuss the clinical and pathological features of retinal degenerations that are important to their potential treatment with stem cell therapy; the unique combination of eye anatomy and imaging capabilities that makes it an excellent target organ for early stem cell therapy in humans; and the status of human trials.

Clinical and Pathological Features of Retinal Degenerations

Retinitis Pigmentosa

Retinitis pigmentosa (RP) is a group of Mendelian hereditary disorders characterized clinically by bilateral progressive loss of peripheral vision, a marked ring-like constriction of the visual field, night blindness, and late loss of central

vision. As a group the population prevalence of RP is about 1:4,000, so the estimates are that approximately 100,000 in the USA have this disease. Investigators have identified at least 45 loci for mutations that can cause retinitis pigmentosa, and these genes collectively account for disease in a little over half of all patients [5–7]. Of the cloned genes for retinitis pigmentosa it is estimated that dominant retinitis pigmentosa account for about 50 %, recessive retinitis pigmentosa account for about 40 % and X-linked retinitis pigmentosa account for approximately 80 % of cases, indicating that many genes remain to be identified [6, 8]. Rods are the predominantly affected photoreceptors and dysfunction causes night blindness and peripheral field loss beginning as early as the teenage years [9]. Disease progression leads to central acuity loss and legal blindness in the majority of patients [10]. Classic findings on funduscopic exam include perivascular bony spicule pigmentation, attenuated arterioles, and waxy optic disc pallor, typically associated with vitreous cells and posterior subcapsular cataracts. However, many of these findings may be absent in early stages of disease [11, 12]. Electroretinogram (ERG) testing is important for diagnosis and may provide prognostic information [10]. The genetics of retinitis pigmentosa are extremely complex with diverse modes of inheritance [12]. Potential interventions include vitamin A therapy and carbonic anhydrase inhibitors, but treatment options are extremely limited in the majority of cases with no effective form of therapy. Results evaluating vitamin A efficacy have shown limited benefit but potential risks exist with oral vitamin A supplementation, including the risk of hepatotoxicity [13]. Carbonic anhydrase inhibitors have shown clinical benefit in reducing macular edema and improving visual acuity in some patients with retinitis pigmentosa [14].

Genetics

The genetics of retinitis pigmentosa are extremely complex with diverse modes of inheritance including dominant, recessive, X-linked, mitochondrial, and digenic forms [12]. The disease may manifest solely with visual symptoms or may be accompanied by a constellation of systemic findings in patients with syndromic retinitis pigmentosa. The diversity in genetic transmission and clinical presentation is not entirely surprising given that retinitis pigmentosa constitutes a broad group of diseases that arises from diverse biological pathways.

Retinitis pigmentosa demonstrates multiple modes of segregation [15]. Autosomal dominant transmission occurs most frequently and accounts for 20 % of retinitis pigmentosa cases. Symptoms are generally less severe with adult-onset with variable penetrance of symptoms. Autosomal recessive disease occurs in 13 % of cases and is characterized by earlier onset of symptoms and severe vision loss. X-linked recessive disease accounts for 8 % of cases and has the poorest visual prognosis with early onset and rapid progression of symptoms [12]. Visual deficits typically present within the first decade of life and progress to partial or complete blindness by the third or fourth decade. In approximately 20 % of nonsyndromic cases, the mode of

transmission cannot be established because of an unclear family history. These cases are termed simplex retinitis pigmentosa and presumed to arise from autosomal recessive or X-linked transmission. Syndromic retinitis pigmentosa, in which vision loss occurs in the settings of extraocular disease manifestations, constitutes 25 % of cases with Usher (10 %) and Bardet–Biedl (5 %) syndromes occurring most frequently [15].

Mutations in 53 genes are known to cause nonsyndromic retinitis pigmentosa or Leber's congenital amaurosis (LCA), which may be indistinguishable from early onset retinitis pigmentosa. This includes 25 genes in autosomal recessive retinitis pigmentosa (arRP), 17 genes in autosomal dominant retinitis pigmentosa (adRP), 13 genes in recessive LCA, 2 genes in dominant LCA, and 6 gene mutations in X-linked retinitis pigmentosa (xlRP) [15]. Mutations in a single gene, such as rhodopsin or neural retina-specific leucine zipper (NRL), may result in multiple forms of disease such as adRP and arRP. The proportion of disease caused by mutations in a particular gene is highly variable [15]. The largest proportion of retinitis pigmentosa is caused by mutations in rhodopsin (RHO) in adRP (26.5 %), Usher syndrome 2A (USH2A) in arRP (10.0 %), retinal guanylate cyclase 2D (GUCY2D) in recessive LCA (21.2 %), and retinitis pigmentosa GTPase regulator (RPGR) in xlRP (74.2 %). A significant proportion of the molecular defects underlying retinitis pigmentosa are known to affect the phototransduction cascade, visual cycle, outer segment structure, cilium-mediated protein trafficking, cellular interaction/adhesion, transcription factors, and RNA-intron splicing factors.

Symptoms and Clinical Findings

Retinitis pigmentosa is phenotypically heterogeneous with wide variation in severity, age of onset, and progression. Classically, retinitis pigmentosa manifests with early night blindness (nyctalopia) beginning in teenage years followed by loss of peripheral visual field. The majority of patients are classified as legally blind by age 60 with central visual field diameters less than 20° [9]. Defects in blue–yellow color perception may occur in advanced stages when visual acuity is 20/40 or worse [16].

Syndromic retinitis pigmentosa is a term used to describe cases of retinitis pigmentosa associated with extraocular symptoms. Approximately 25 % of retinitis pigmentosa cases are syndromic and over 30 forms have been identified [17]. Usher syndrome is the most common form and is associated with sensorineural deafness. It accounts for about 10 % of retinitis pigmentosa cases and is divided into three major groups. Type 1 demonstrates profound congenital deafness, vestibular symptoms, and childhood-onset retinopathy [18]. Type 2 manifests with congenital partial, nonprogressive deafness, absence of vestibular symptoms, and mild later-onset retinopathy [19, 20]. Type 3, the least common form, demonstrates progressive deafness beginning in the third decade and adult-onset retinopathy [21]. Bardet–Biedl syndrome is the second most common form of syndromic retinitis

pigmentosa and accounts for 5 % of retinitis pigmentosa cases [15]. It is associated with polydactyly, obesity, renal dysfunction, and mental retardation. Other forms of syndromic retinitis pigmentosa account for 10 % of all retinitis pigmentosa cases and include Refsum's disease, Bassen–Kornzweig syndrome, Kearne–Sayre syndrome, Batten's disease, and Senior–Loken disease. A complete listing of genes implicated in retinitis pigmentosa can be found on the Retinal Information Network web site http://www.sph.uth.tmc.edu/retnet/.

Retinitis pigmentosa classically leads to fundus changes with accumulation of bony spicule pigmentation. Lesions are generally perivascular and localized to the mid-periphery where rods are concentrated. However, pigment distribution is often variable and may be diffuse, sectoral, or even be absent in certain subtypes of retinitis pigmentosa. Other signs include abnormal retinal pigmentation changes, attenuated arterioles, vitreous cells, waxy optic disc pallor, and blue–yellow color vision deficiency. Vitreous cells and opacities are the most consistent characteristics across all forms of retinitis pigmentosa. Notably, early stages of retinitis pigmentosa may lack appreciable funduscopic findings [5, 11, 12]. Retinitis pigmentosa patients, particularly those over age 40, may demonstrate cystoid macular edema, epiretinal membranes, diffuse retinal vascular leakage, macular preretinal fibrosis, macular RPE defects, and posterior subcapsular cataracts. Other associated findings include myopia and astigmatism [5, 11, 12, 22–24].

Treatment

Treatment options are extremely limited for most retinitis pigmentosa subtypes with no effective approach for prevention, stabilization, or reversal of visual loss.

The efficacy of vitamin A and E supplements on slowing retinitis pigmentosa progression was examined in a randomized, double-masked, prospective study [13]. About 601 patients with non-syndromic retinitis pigmentosa and Usher syndrome (type 2) were randomized into four treatment groups receiving 15,000 IU/d of vitamin A, 400 IU/d of vitamin E, 15,000 IU/d of vitamin A plus 400 IU/d of vitamin E, or trace amounts of both vitamins and followed for 4–6 years. The trial concluded that (1) vitamin A groups demonstrated slower rates of decline in cone ERG amplitudes (2) vitamin A groups were 32 % less likely to have a decline in ERG amplitude of 50 % or more from baseline (3) vitamin E groups were 42 % more likely to have a decline in ERG amplitude of 50 % or more from baseline, and (4) there was no significant difference in visual acuity and field loss. The reduction of ERG decline in patients receiving vitamin A was limited to the 30 Hz and 0.5 Hz flash amplitudes. Significantly, these patients did not demonstrate any improvement in psychophysical visual parameters [25, 26].

Thus, these results suggest that benefits of vitamin A therapy are limited and must be weighed against potential risks such as teratogenic effects in pregnant women, elevated intracranial pressure, hepatomegaly, bone disease in young individuals, and elevated serum lipids [27–29]. Currently many practitioners do

not use vitamin A supplementation routinely due to the small treatment effect and the need for monitoring of vitamin A toxicity. In addition, the mixed molecular etiology of retinitis pigmentosa suggests that response to vitamin A may vary across retinitis pigmentosa subtypes. Studies in ABCA4 knockout mice demonstrated increased rates of lipofuscin deposition and photoreceptor degeneration in mice on vitamin A supplementation. These results suggest that if vitamin A supplementation is employed, it should be done so selectively [30, 31] as it may have a deleterious effect on certain subsets of retinitis pigmentosa patients. Because of the small magnitude of the effect on ERG, lack of improvement in psychophysical parameters, concerns about toxicity, and the varied genetics of retinitis pigmentosa, the use of vitamin A supplementation to slow retinitis pigmentosa progression has not been universally adopted. If patients are placed on oral vitamin A therapy, they should undergo periodic liver function testing, osteoporosis screening, and fasting serum vitamin A measurements to avoid toxicity.

Other therapies have also been advocated as potentially effective in retinitis pigmentosa. To date, however, there is no evidence of clinical visual improvement with lutein supplements [32], docosahexaenoic acid supplements [33–35], light deprivation [36], therapeutic bee stings [37], vasodilators [38], or placental tissue injections [39]. Interestingly, repeat intravitreal injections and/or pars plana vitrectomy are not currently used to treat patients with retinal degenerations, despite the fact that there is a well-known rescue effect of vitreous and subretinal surgery on retinal degeneration. Subretinal insertion of a dry needle results in a degree of photoreceptor rescue similar to that of intravitreal or subretinal basic fibroblast growth factor injection in the Royal College of Surgeons rat [40]. Anterior chamber injection of placebo and brain-derived neurotrophic growth factor produces similar rescue effects in axotomized rat ganglion cells [41]. Lensectomy and vitrectomy alone rescue degenerating photoreceptors in the P347L transgenic pig, which contains a rhodopsin mutation known to cause retinitis pigmentosa in humans [42]. Subretinal saline injection produces a rescue effect in the Royal College of Surgeons rat [43]. These studies demonstrate clearly that vitreous and subretinal surgery alone may produce some rescue effect in retinal degenerations, but long-term demonstration of their efficacy awaits additional preclinical and clinical trials.

There is some therapeutic benefit of dietary modifications and nutritional supplements for two rare forms of syndromic retinitis pigmentosa. Phytanic acid oxidase deficiency (Refsum's disease) arises from failure of phytanic acid degradation and consequent elevation of serum phytanic acid. Clinical manifestations include ataxia, peripheral neuropathy, deafness, and cardiac conduction defects [44–46]. Dietary restriction of phytanic acid may halt or reduce progression of retinitis pigmentosa. Abetalipoproteinemia (Bassen–Kornzweig syndrome) is characterized by low serum levels of apolipoprotein B, resulting in fat malabsorption and low plasma concentrations of fat-soluble vitamins. Systemic signs generally manifest in childhood and include diarrhea, cerebellar ataxia, and acanthocytosis. Therapy with high doses of vitamin A may allow rapid restoration of visual function in early stages of disease [47–49]. Laboratory studies of serum phytanic acid levels and serum lipoprotein electrophoresis can assist in the diagnosis of Refsum's disease and Bassen–Kornzweig syndrome, respectively.

Visual function in retinitis pigmentosa may be improved by monitoring for and treating associated conditions such as cystoid macular edema, posterior subcapsular cataract, and epiretinal membranes. In addition, referral to low vision clinics can help optimize remaining visual function.

The Argus II Retinal Prosthesis System developed by Second Sight Medical Products, Inc. is intended to provide electrical stimulation of the retina to elicit visual perception in blind subjects with retinitis pigmentosa [50]. The technology is currently being evaluated in a clinical study conducted in the USA and recently received a CE (European Conformity) mark in Europe which is a key indicator of a product's compliance with European Union legislation. The device consists of a surgically implanted 60-electrode stimulating microelectrode array consisting of 200 μm diameter disc electrodes, an inductive coil link used to transmit power and data to the internal portion of the implant, an external belt-worn video processing unit and a miniature camera mounted on a pair of glasses [51, 52]. The video camera is designed to capture a portion of the visual field and relay the information to the video processing unit. The video processing unit then digitizes the signal in real time, applies a series of image processing filters, down-samples the image to a 6×10 pixelized grid, and creates a series of stimulus pulses based on pixel brightness values and look-up tables customized for each subject [51]. The stimulus pulses are delivered to the microelectrode array via application-specific circuitry and a superior-temporally placed inductive radio frequency coil link allowing for wireless forward and reverse telemetry between intra and extra-ocular portions of the system [51]. The prosthesis is expected to generate limited amounts of vision in patients with severe to profound vision loss in the range of hand motions or light perception vision.

Age-Related Macular Degeneration

Age-related macular degeneration (AMD) affects 30–50 million elderly people worldwide and is the leading cause of blindness in individuals over the age of 50 in the Western world [53, 54]. It is estimated that approximately 30 % of adults over the age of 75 have some signs of AMD and that at least 10 % develop the advanced or late stage of disease [55, 56]. AMD as a disease entity primarily exists in two forms, nonexudative (atrophic or dry) AMD and exudative (neovascular or wet) AMD. Although the vast majority of patients with AMD are of the nonexudative type, approximately 90 % of significant vision loss due to AMD is secondary to central vision deterioration from the exudative type [56, 57]. Early in the course of disease there is cellular dysfunctional without cell death. In late-stage disease, AMD is characterized by extensive cell death, as with late-stage RP.

Genetics of AMD

Age-related macular degeneration is a complex disease that results from a combination of genetic and environmental factors. Many of these factors have been

identified, but some remain unknown. Because AMD occurs late in life, it has been very difficult to elucidate the genetic factors correlated with the disease. AMD's heterogenicity in phenotypes presents a challenge as well [58]. It also may be discovered that each individual's susceptibility is due to multiple genetic and environmental effects and interactions [58–62].

Symptoms and Clinical Findings

Patients with advanced AMD typically present with blurry central vision, metamorphopsia, and reduced vision. These symptoms can then evolve to a central scotoma and severe loss of vision [63]. Ophthalmoscopic examination of the fundus at late stages of disease demonstrates patchy chorioretinal atrophy in the dry type and exudation in the wet variety, often manifested by the presence of retinal hemorrhages and lipid exudate in and around the macula [63].

One of the earliest clinical findings associated with AMD is the presence of drusen, which represent accumulation of extracellular material beneath the RPE [64]. In the case of dry AMD, loss of vision develops due to loss of the RPE, photoreceptors, and/or choriocapillaris; this can lead to patches of atrophy which are manifest clinically by central and paracentral scotomas [64]. In the case of wet AMD overexpression or loss of normal apical–basal polarity in the expression of angiogenic factors such as vascular endothelial growth factor (VEGF) can cause neovascularization to arise from the neural retina (retinal angiomatous proliferation) or choriocapillaris. In early stages of the disease patients experience minimal vision loss but some symptoms may occur such as blurred vision, visual scotomas, decreased contrast sensitivity, abnormal dark adaptation, and the need for bright light or magnification to decipher images [64]. In the late stages of advanced non-neovascular disease, patients typically present with a gradual loss of vision that becomes more severe and affects central or pericentral vision [64]. This form usually progresses and leads to irreversible vision loss. In patients with neovascular disease, loss of vision can be much more sudden with loss occurring within days to weeks due to subretinal hemorrhage or fluid accumulation secondary to choroidal neovascularization [64, 65].

Treatment

While the last decade has brought about a revolution in the treatment of exudative AMD, there are currently no approved therapies for geographic atrophy. Numerous investigational therapies are in various stages of clinical trials. These include ciliary neurotrophic factor, complement inhibitors, weekly vaccination with glatiramer acetate, fenretinide and OT-551 [66–70]. These therapies are promising, but none have progressed beyond clinical trials, leaving a large void in the current therapy of geographic atrophy.

Ninety percent of AMD patients who experience severe vision loss do so as a result of choroidal or intraretinal neovascularization [71]. Choroidal neovascularization represents growth of neovascular tissue from the choriocapillaris, within Bruch's membrane, and eventually in the subretinal pigment epithelium and/or subretinal space. Retinal angiomatous proliferation is a form of wet AMD in which the abnormal vessels arise from the neural retina [72, 73]. Developing new treatments that prevent or reverse vision loss in AMD are of paramount importance due to the severe visual deficits that occur with this condition and the knowledge that disease prevalence will increase with shifting demographics of an aging western population.

Treatments for the wet form of this disease involve intravitreal antiangiogenic therapy, photocoagulation and photodynamic therapy, and vitreoretinal surgery. Intravitreal antiangiogenic treatment is currently the primary therapy for wet AMD and delivered directly to the vitreous. Treatment with intravitreal injection of anti-VEGF agents improves vision in patients with wet AMD but maintenance of the therapeutic effect requires continued administration of intravitreal agents, and this can be associated with potentially serious side effects such as endophthalmitis, retinal detachment, intraocular hemorrhage, increased intraocular pressure, and, in some cases, retinal detachment [74]. Photodynamic therapy uses light sensitive medicine that identifies abnormal vessel growth under the macula. Laser light then activates the light sensitive dye which can then decrease exudation from the neovascularization.

Despite these significant advances in the management of exudative AMD, there is a large unmet need for many patients with this condition. More than 50 % of patients do not respond to therapy with anti-VEGF drugs, and many patients with advanced disease have loss of vision due to scar formation and altered subretinal architecture. These limitations have led to the investigation of alternative treatment modalities for subfoveal exudative AMD, including subfoveal membranectomy with and without RPE transplantation or translocation [75–79] and macular translocation [80]. Initial efforts to improve vision with cell transplantation alone have not been met with success; reconstitution of the normal subretinal architecture is necessary for visual improvement in these individuals. Ultimately this will require reconstruction of macular anatomy in patients with advanced vision loss in exudative AMD [81]. Successful maculoplasty will require replacing or repairing damaged cells (using transplantation, translocation or stimulation of autologous cell proliferation); immune suppression (if allografts are used to replace damaged cells); and reconstruction or replacement of Bruch's membrane (to restore the integrity of the substrate for proper cell attachment). Successful maculoplasty will build on prior development of surgical techniques for managing severe vision loss in AMD patients with advanced subfoveal exudation. These techniques include surgical excision of choroidal neovascularization; [75–79, 82–84] surgical excision combined with allograft transplantation of adult or fetal RPE [85–96] or iris pigment epithelium [97–108] or macular translocation with or without choroidal membrane excision [109–127].

Simple excision of the subfoveal neovascular membrane in AMD leaves a large RPE defect under the fovea due to the removal of native RPE along with the surgically removed neovascular complex [128]. Resulting persistent RPE defects lead to the development of progressive choriocapillaris and photoreceptor atrophy [129]. Histopathology after subfoveal membranectomy alone shows absence of large swatches of native RPE, combined with damage to the outer retina, choriocapillaris atrophy and absence and/or damage to the inner aspects of native Bruch's membrane [130, 131]. The status of host Bruch's membrane has a profound effect on the behavior of RPE transplanted after subfoveal membranectomy [81, 132–139]. Thus reconstruction of Bruch's membrane is a necessary component for successful maculoplasty [140]. Given the issues with the status of host Bruch's membrane, and the paucity or absence of native RPE and/or photoreceptors in advanced disease states, there is a need for a combined approach with cell replacement therapy and Bruch's membrane reconstruction that will be required to reverse vision loss in these advanced disorders. There are significant logistical challenges to cell replacement therapy in this disease, including the need for large numbers of cells needed for cell replacement, and the need for immune suppression if allo grafts are used for transplantation. Transplantation of intact sheets and suspensions of primary RPE cells have been previously attempted in humans, with mixed results in terms of graft survival and improvement in vision [85, 91, 141–144]. Stem cells are an ideal replacement source for these lost or damaged cells, since stem cells have a significant ability to proliferate in vitro prior to transplantation and in vivo after subretinal transplantation.

Unique Combination of Anatomy and Imaging Capabilities that Make the Eye an Excellent Choice for Stem Cell Therapy

It is no accident that the eye has become one of the first organs to be treated with stem cell therapy in humans, as the eye is an excellent target organ for stem cell therapy [141]. There are several reasons for this, including the facts that retinal degenerations are well characterized, and excellent animal models exist for many of these diseases. In addition, the eye is optically transparent, so that the transplant site can be monitored directly with slit lamp biomicroscopy, indirect ophthalmoscopy, fundus photography, auto fluorescence imaging, fluorescein angiography, and optical coherence tomography, which gives us advantages of in vivo "histological sections" through a transplant area. In addition there is excellent function testing, including visual fields testing, multifocal electroretinogram (ERG), and microperimetry.

Autofluorescence imaging is a technique that allows for topographic mapping of fluorescence emanating from the retina, retinal pigment epithelium, and choroid in health and disease [145, 146]. In this technique fluorescence-based images of the human fundus are captured using different combinations of excitation and barrier filters, allowing the ophthalmologist to discern the topographic distribution of various fluorophores in the retina and deeper layers (Fig. 1.1). Many fluorophores

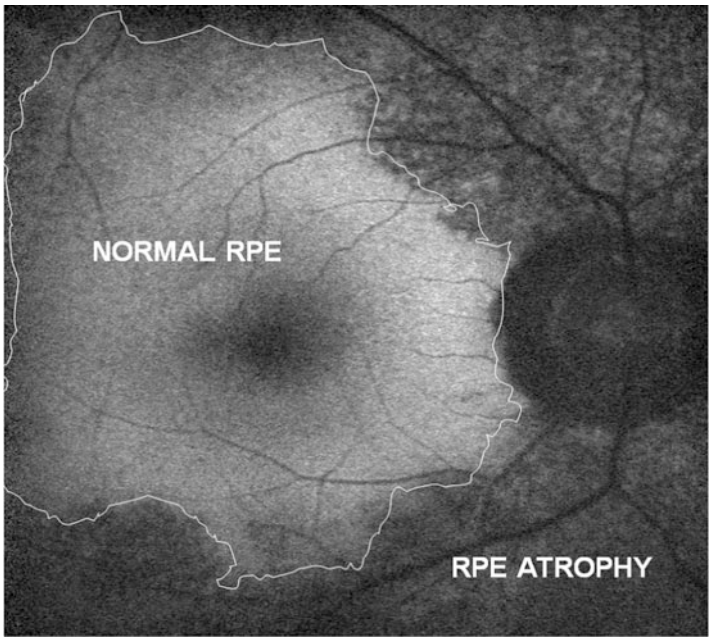

Fig. 1.1 Autofluorescence imaging of the retinal pigment epithelium (RPE), demonstrating the topographic distribution of fluorescence in the posterior segment of the human eye. Many fluorophores are contained within RPE, so that areas of normal RPE may exhibit autofluorescence compared to dark areas of patchy RPE atrophy

are contained within RPE cells, and thus the retinal pigment epithelium will fluoresce using this type of imaging, allowing for determination of areas of RPE absence and areas of atrophic patches consistent with areas of geographic atrophy (Fig. 1.1). As these techniques develop further, it is important to recognize a priori that there is no reason to think that all fluorophores will be contained within the RPE. For example, we have previously demonstrated the presence of nitro-A2E within human Bruch's membrane in elderly individuals [147], and several authors have reported decoupling of the auto fluorescent signature of A2E from the overall autofluorescent signature emanating from the human RPE. It is likely that the information obtained from autofluorescence imaging of the human retina will increase dramatically with improvement in the excitation sources and detection systems. For stem cell transplantation, autofluorescence imaging may allow us to detect the reconstruction of the RPE monolayer, if the transplanted stems cells incorporate A2E or other fluorophores after subretinal transplantation.

Fluorescein angiography (FA) is a technique used to image blood flow of the retina and choroid by using sequential fluorescence imaging following the intravenous injection of sodium fluorescein (Fig. 1.2). Histopathology studies have revealed that there is an accumulation of autofluorescent material in the retinal pigment epithelium as well as autofluorescent deposits of extracellular material in macular and retinal disease [146]. Use of fluorescein angiography allows for the

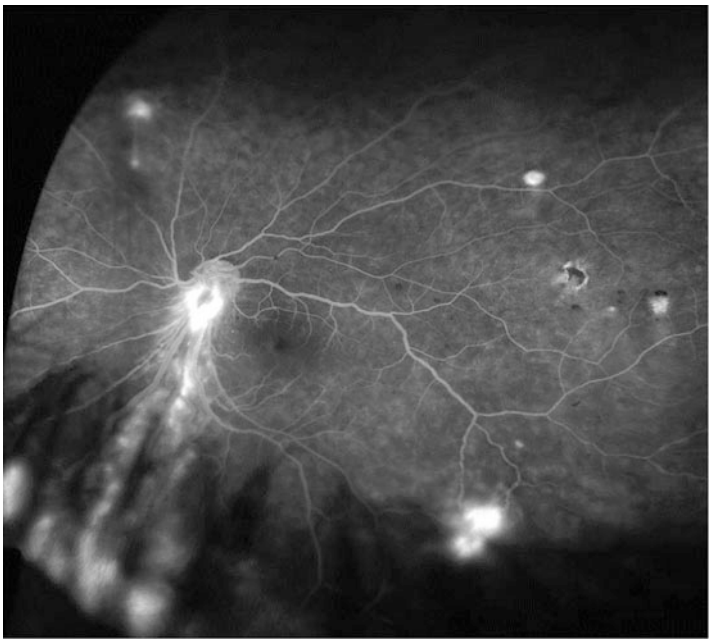

Fig. 1.2 Wide field retinal angiography to image blood flow of the retina and choroid obtained after intravenous injection of sodium fluorescein. In addition to monitoring for non-perfusion, angiography can be used to detect angioma (shown here) or vascular leakage, which can be a sign of graft rejection

visualization of atrophic patches that appear well-demarcated, hyperfluorescent areas due to loss of retinal pigment epithelium [148]. These cells, if intact, would otherwise weaken transmission.

Indocyanine green (ICG) is a cyanine dye that allows for enhanced imaging patterns of circulation when compared to fluorescein dye given a spectral absorption between 805 and 835 nm [149]. As with fluorescein dye, indocyanine allows for the visualization of atrophic areas of degeneration. Indocyanine green has primarily been used in the diagnosis and interpretation of occult choroidal neovascularization in age-related macular degeneration and for identification of angiomatous lesions of the retina and polyps in the choroid [150]. The unique properties of indocynanine green allows for visualization of macular dystrophies through overlying pathologic conditions such as hemorrhage, serous fluid, lipid, and pigment [151]. Indocyanine green has been utilized as an adjunct tool along with fluorescein angiography for the diagnosis of age-related macular degeneration [151].

Angiography allows the ophthalmologist to discern the perfusion status of the retina and choroid, as diseases that impair perfusion can be diagnosed on the basis of abnormalities in the dye filling pattern on sequential angiogram photos. In addition to monitoring for non-perfusion, FA and ICG angiography can be used

to monitor for vascular leakage, which can be diagnosed on the basis of accumulation of extracellular dye due to increased vascular leakage. There are several reasons why this is particularly useful to the field of stem cell transplantation. First and foremost, in the absence of native RPE there is secondary non-perfusion of the choriocapillaris, and this non-perfusion is evident on both fluorescein and ICG angiography. Animal studies suggest that the choriocapillaris can reperfuse after replacement of the RPE, and thus angiography can be used to monitor the outer retinal blood supply for the success of transplanted cells. Both techiques allow for the monitoring of vascular integrity (Fig. 1.2) [150, 152]. In addition ICG and FA allow the ophthalmologist to detect leakage of dye, which can be a sign of graft rejection.

Optical coherence tomography (OCT) is an imaging method currently in widespread clinical use that provides in vivo images from the human retina. OCT relies on differences in the index of refraction of ocular tissue to generate a cross-sectional image of the retina and the vitreoretinal interface. OCT can be used to measure foveal and extrafoveal retinal thickness and can be used to determine the thickness of the outer nuclear layer and integrity of the outer segment–inner segment junction. OCT can be used to detect RPE atrophy and outer retinal atrophy and to determine the thickness of the nerve fiber layer. In geographic atrophy, OCT can reveal atrophy of the choriocapillaris, particularly with enhanced depth choroid imaging. In the study done by Neurotech on ciliary neurotrophic factor (CNTF), OCT was used to demonstrate increasing thickness of the outer retina in patients with nonexudative AMD who received the CNTF-releasing implant [66]. In principle, OCT can be used to monitor the ability of transplanted stems cells to repopulate Bruch's membrane, the return of retinal thickness back to normal, and reestablishment of choroid thickness in patients with atrophy. OCT can also be used to determine adverse events after transplantation, including the development of retinal edema, after treatment of patients with exudative and nonexudative age-related macular degeneration (Fig. 1.3) [141, 153, 154].

In addition to these structural studies, there are several excellent functional tools for determining retinal function in eyes of retinal degenerations treated with stem cells. Microperimetry can assess macular sensitivity and retinal fixation by providing a retinal visual function map on a selected, localized fundus location with preset or customized scan patterns (Fig. 1.4a, b) [155]. In this technique, the retina is stimulated by illumination with small spot sizes under direct visualization; this allows the examiner to discern the retinal sensitivity as a function of illumination level and spot size in areas of the retina affected by retinal degenerations, and in treated and control regions. In principle, a beneficial effect of transplantation would be manifested by increasing retinal sensitivity on microperimetry.

Similarly, multifocal ERG can also be used to determine a decrease in retinal function due to disease, and an improvement in dysfunction after retinal transplants. Multifocal ERG allows for a topographical measure of electrical activity in distinct areas of the retina (Fig. 1.4c) [156]. Multifocal ERG can stimulate multiple retinal areas at the same time and detect each response independently [157]. In retinal

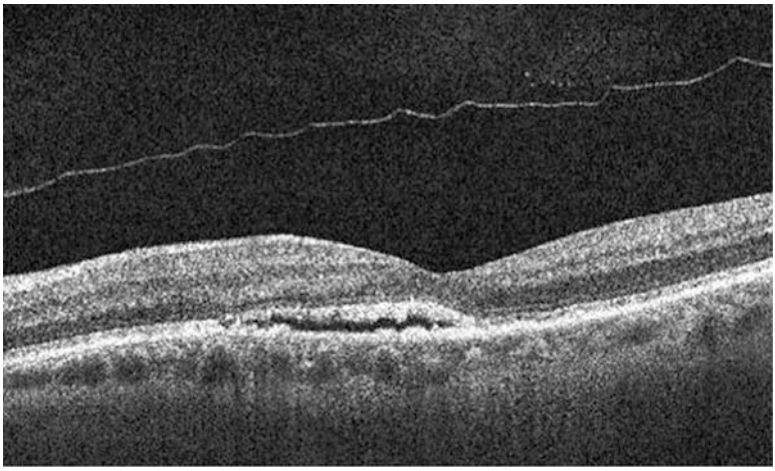

Fig. 1.3 Optical coherence tomography (OCT) reveals a small pocket of subretinal fluid in an asymptomatic patient with age-related macular degeneration. OCT can be used to measure the thickness of the outer nuclear layer and integrity of the outer segment–inner segment junction after successful cell transplantation

degenerations, there is typically a decrease in amplitude, or absent ERG signal, in areas of retinal dysfunction; this change is often present only in an area of dysfunction. There is an improvement in the global ERG in animals with retinal degenerations receiving transplants of stem cells (Fig. 1.5) [158]. In principle, improvement in retinal function after stem cell transplantation should be topographic and result in a focal improvement in ERG in the area of the transplant. These advantages in imaging and focal detection of function should not be overlooked, since cell transplants in other areas of the body do not have similar advantages. These advantages present a unique opportunity to detect the beneficial effects of stem cells in the treatment of retinal diseases. Their use also makes diseases such as age-related macular degeneration an attractive option to begin clinical trials with stem cells. The ranges of clinical and diagnostic tools also help provide the necessary efficacy and safety data to move trials forward [159].

Status of Efforts to Differentiate Stem Cells into Photoreceptors and RPE

As a general principle, patients who could directly benefit from cell-based therapies with retinal degenerative disease such as RP and AMD will require replacement of lost photoreceptor cells, RPE, and possibly choriocapillaris [160]. Korte et al. have shown the choriocapillaris can regenerate if areas of absent RPE can be repopulated with new RPE. Thus the clinical need here is to promote the differentiation of stem cells into photoreceptors (rods and cones) and RPE.

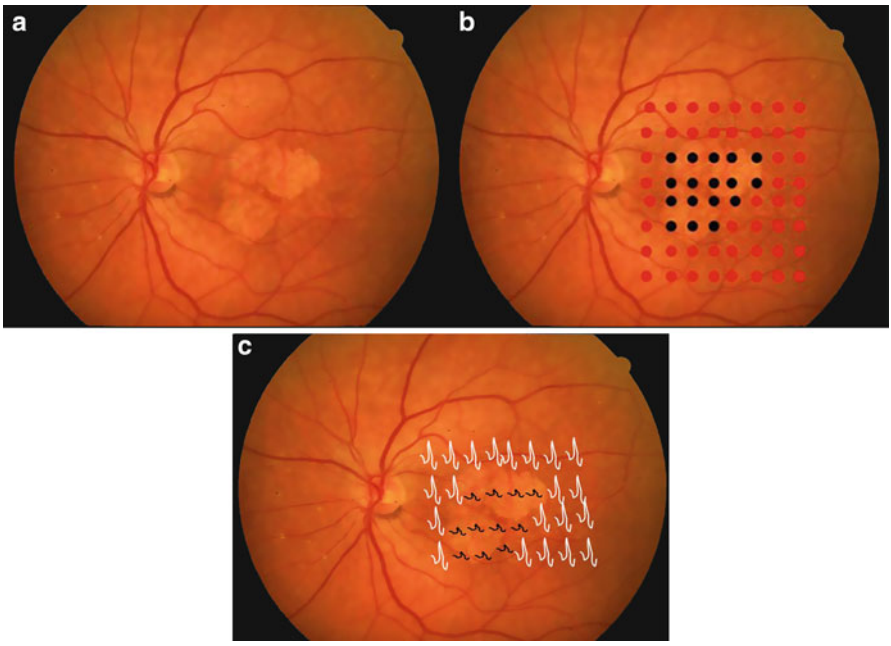

Fig. 1.4 (**a**) Microperimetry can be used to assess macular sensitivity and retinal fixation in normal and atrophic areas of retina by providing a retinal visual function map on a selected, localized fundus location with preset or customized scan patterns. (**b**) In this technique, the retina is illuminated with small spot sizes under direct visualization; this allows the examiner to demonstrate decreased retinal sensitivity in regions of geographic atrophy (*Right panel*, *black circles*) and normal sensitivity in adjacent regions (*Right panel*, *red circles*). (**c**) Multifocal electroretinography (mERG), which allows for topographical measure of electrical activity in distinct areas of the retina, can be used to monitor disease progression and efficacy of therapy. In retinal degenerations, there is typically a decrease in amplitude, or absent ERG signal, in areas of retinal dysfunction (*black tracings*)

In dry AMD, loss of vision arises from loss of RPE and photoreceptors with secondary atrophy of choriocapillaris. A potential treatment for AMD and inherited disease that affect the RPE and photoreceptors would be cell replacement therapy, but one significant hindrance to the clinical use of cell transplantation for treatment of retinal degenerations is the availability of a source of replacement cells. Although RPE derived from prenatal and postnatal tissue has been isolated and induced to grow in vitro, such sources are limited and vary in terms of quality and expansion capacity [141, 161–163]. Moreover, it has been demonstrated that postmitotic photoreceptor precursor cells can be derived from tissue of the early postnatal mouse retina (P1–P5) [164, 165]. However, equivalent retinal cells in humans would have to be derived from second-trimester fetuses. While these studies provide solid evidence that transplantation strategies show great potential, an approach such as this would have ethical implications as well as the problem of a limited reservoir of donor cells [165].

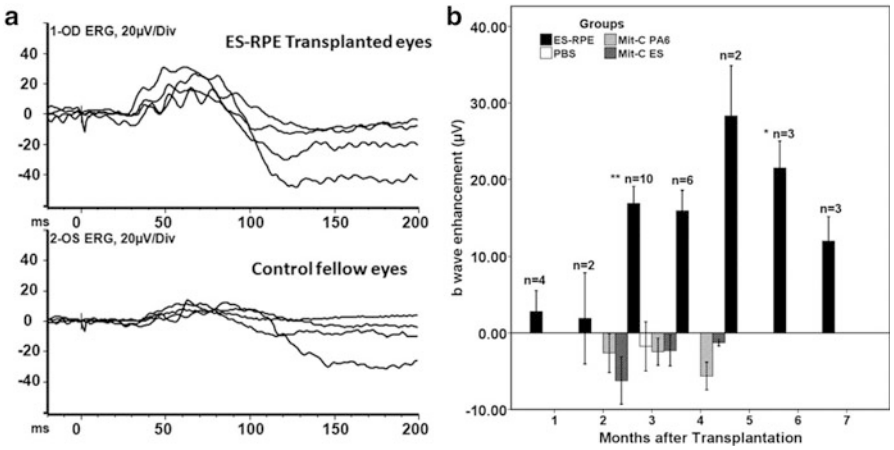

Fig. 1.5 ERGs of *Rpe65*^{rd12}/*Rpe65*^{rd12} mice after subretinal transplantation with ES cell-derived retinal pigment epithelial (RPE)-like cells confirm functional rescue. (**a**) ERG from mice after 3 months transplantation. Eyes transplanted with ES cell-derived RPE-like cells (*upper*) showed higher b-wave amplitudes compared with control fellow eyes (*lower*). Traces represent readings from different mice. (**b**) b-wave enhancement in mice 1–7 months post-transplantation, as indicated by *black solid bars*. b-wave enhancement is defined as the difference in maximum ERG responses of transplanted and control fellow eyes (μV). Unpaired *t* tests were performed for paired differences in b-wave peaks between transplanted and control eyes. At 3 and 6 months post-transplantation, ERGs from transplanted eyes show a statistically significant rescue effect (**$P = 0.001$ and *$P = 0.038$, respectively). Although the difference was not statistically significant at 4, 5, and 7 months after transplantation, the b-wave amplitudes in the transplanted eyes were consistently higher than the control fellow eyes. The number of mice analyzed per time point is indicated. ERGs were performed on both eyes (injected and control) simultaneously. There is no statistically significant difference between injected and control eyes in the other three control groups. *White bar*, b-wave enhancement in PBS injected mice; *light-shaded bar*, b-wave enhancement in mitomycin-C treated PA6 cell transplanted mice; and *dark-shaded bar*, b-wave enhancement in mitomycin-C treated undifferentiated ES cell transplanted mice. *ES-RPE* ES cell-derived RPE-like cells, *Mit-C* mitomycin-C, *ES* embryonic stem, *PBS* phosphate-buffered saline. Reproduced from Wang et al. [158]

Stem cells are an excellent source of cells for replacement therapy given the limited reservoir of donor cells for RPE replacement strategies, lack of regeneration of photoreceptors, and variation in success of autografts. Stem cells have been isolated from a variety of sources including embryo and adult eye [164, 166]. Human embryonic stem cells (hESC) are being investigated as a potential source of photoreceptors and RPE and are promising candidates for therapeutic use.

As mentioned, the strategy in cell replacement therapy using stem cells is to differentiate these cells into photoreceptors or RPE. The extracellular environment plays a critical role in the differentiation of stem cells into the target cell type and extracellular matrix can differentiate cells into the adjacent cell layer. For example, hESCs cultured on a monolayer of cells derived from mouse calverium can be induced to a neural fate and express neural progenitor markers such as paired boxed protein (PAX)-6, neurofilament, and glial fibrillary acidic protein (GFAP) (Fig. 1.6) [167]. Similarly, hESCs cultured on a monolayer of RPE (ARPE19) cells

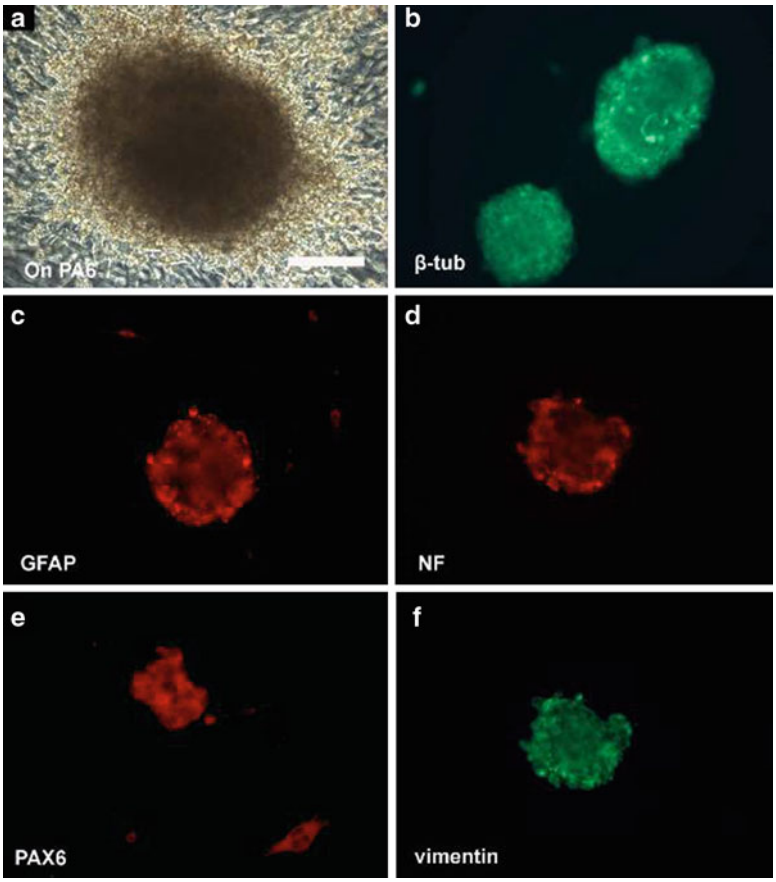

Fig. 1.6 Expression of neural progenitor markers after culturing human embryonic stem cells on mouse PA6. (**a**) Human embryonic stem cells became multilayered and formed pigmented spheres after culturing on mouse PA6 cells for 13 days. Immunofluorescence staining of the spheres demonstrated the presence of several neural progenitor markers including β-tubulin III (>88%) (**b**), GFAP (**c**), neural filament NF200 (>90%) (**d**), PAX6 (>88%) (**e**) and vimentin (**f**). *Bar* = 100 μm

can be induced to express neural retinal markers such as vimentin, neurofilament, and cone–rod homeobox (CRX), which is essential in early photoreceptor development (Fig. 1.7) [167]. Varying extracellular matrix environs such as laminin, matrigel, and/or vitronectin and fibronectin can also induce embryonic stem cells toward a neural progenitor or RPE fate. Embryonic stem cells cultured on laminin, vitronectin, and fibronectin can be induced to express neural progenitor markers such as neurofilament and neural retina-specific leucine zipper (NRL), an intrinsic regulator of photoreceptor development (Fig. 1.8). These cells can also be induced to express RPE markers such as tight junction protein ZO-1 and bestrophin when cultured on

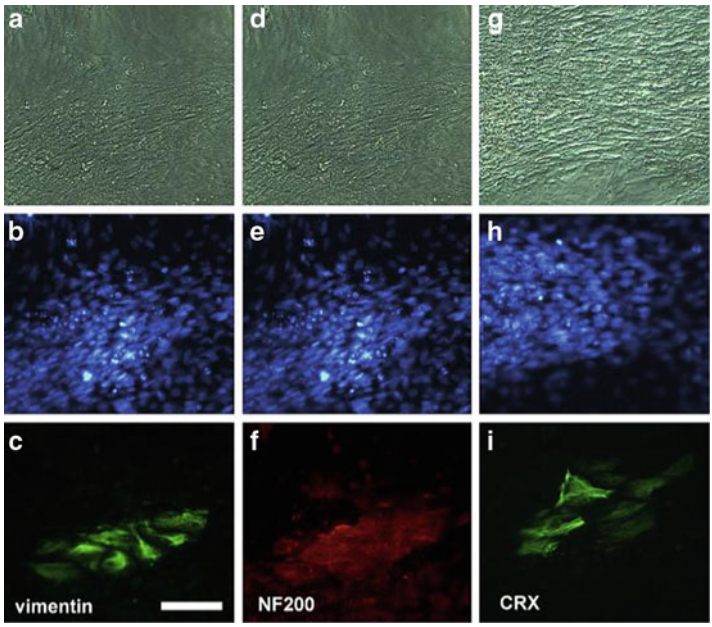

Fig. 1.7 Generation of retinal precursors from neural progenitors after culturing human embryonic stem cells on ARPE19 cells for 10 days. *Top row*, phase-contrast micrographs; *middle row*, nuclei in both ARPE19 and progenitors stained with DAPI. Progenitors expressed neural progenitor marker vimentin (C) and neural filament 200 (F) and photoreceptor-specific protein CRX (I), which is essential during early photoreceptor development. *Bar* = 50 μm

matrigel (Fig. 1.9). Stem cells grown on human Bruch's membrane have also been induced to differentiate to RPE (Fig. 1.10) [167].

Current Status of Human Stem Cells in Clinical Trials

Current clinical trials are underway to evaluate the potential of stem cell therapy in humans. Advanced Cell Technology, Inc. initiated a phase 1 clinical trial in humans in 2011 for the treatment of retinal degenerative disorders [141]. This is the first FDA approved trial for the treatment of macular degeneration using RPE derived from human embryonic stem cells. RPE cells were derived from a single donor human embryonic stem cell line. The preliminary report details the phase 1 trial being conducted to test the safety and tolerability of hESC-RPE in patients with advanced-stage Stargardt's macular dystrophy and dry age-related macular degeneration. Briefly, a human embryonic stem cell (MA09) line was first used to generate hESC-derived RPE, which were then characterized and tested for pathogen contamination. After pars plana vitrectomy, submacular injections of 50,000 cells were used to treat the patient. Patients were immune suppressed with low-dose

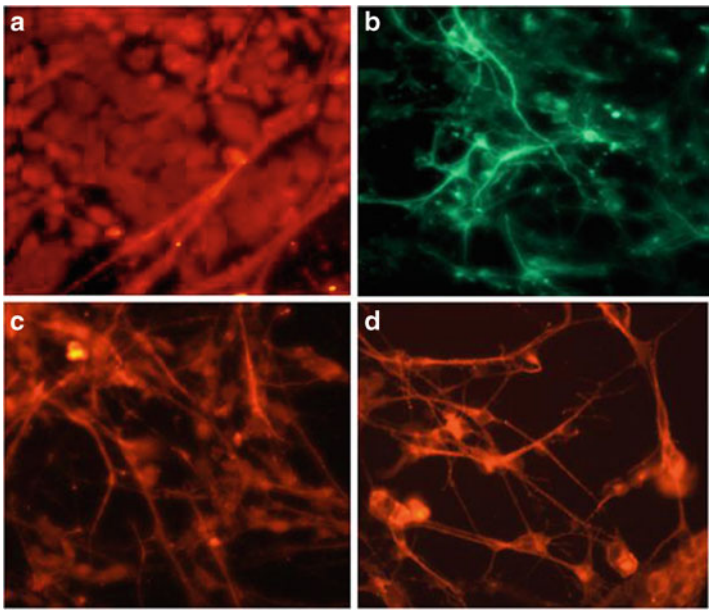

Fig. 1.8 Expression of neural progenitor markers after culturing mouse embryonic stem cells on poly-D-lysine, laminin, vitronectin, and fibronectin. Cells expressed photoreceptor marker NRL (**a**) and neural progenitor markers β-tubulin (**b**) neurofilament 200 (**c**) and vimentin (**d**)

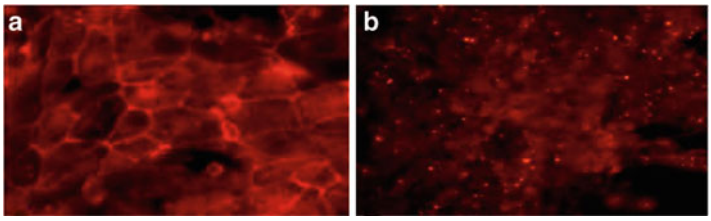

Fig. 1.9 Expression of retinal pigment epithelium (RPE) markers after culturing mouse embryonic stem cells on matrigel. Cells expressed RPE makers Z0-1 (**a**) and Bestrophin (**b**)

tacrolimus and mycophenolate mofetil 1 week before surgery and continued for 6 weeks postsurgery. After 6 weeks patients discontinued tacromilus and continued a mycophenolate regimen for 6 more weeks. Both patients tolerated the injection without signs of postoperative inflammation, rejection, or tumorigenicity at time of the report (4 months follow-up). In the patient with Stargardt's macular dystrophy, transplanted cells attached to Bruch's membrane and persisted throughout the observation period; there was possible visual improvement in the injected eye as shown by visual acuity and Goldmann visual field test. In the AMD patient no clinically detectable sign of successful transplantation was observed, although the patient did not comply with the immunosuppressive drug regimen [168]. Interestingly, there was mild visual improvement in both eyes of the patient with AMD as

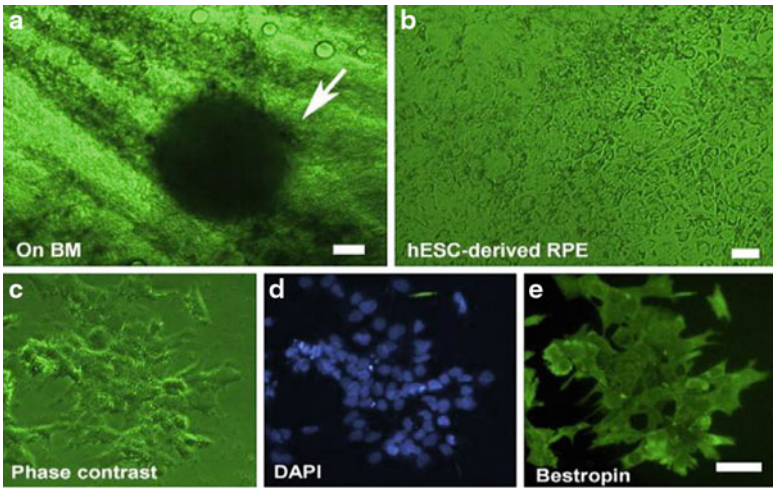

Fig. 1.10 Induction of RPE markers in human embryonic stem cells cultured on human Bruch's membrane. (**a**) Cluster of pigmented human embryonic stem cells 4 days after growing on human Bruch's membrane explants (*arrow*). (**b**) Phase-contrast micrograph of flattened pigmented epithelium-like cells on human Bruch's membrane. (**c**)–(**e**) hESC-derived RPE under phase contrast, DAPI and Bestrophin staining, respectively. *Bar* = 50 μm in (**a**), (**c**)–(**e**); *bar* = 20 μm in (**b**)

shown by visual acuity and Goldmann visual field test. It is unclear whether these visual improvements are due directly to the cell transplant or a secondary cause such as the immunosuppressive drugs or a placebo effect [141]. Nevertheless, these important studies demonstrate that stem cells can be transplanted into the subretinal space in humans without abnormal proliferation, teratoma formation, graft rejection, or other untoward pathological reaction or safety signal.

These short-term results in only two patients, are preliminary but provide valuable proof-of-concept evidence for future treatment of macular degeneration and Stargardt's disease in humans. Long-term follow-up in a larger cohort of patients is needed to draw more meaningful conclusions from this trial [168]. Further trials are also needed to determine the optimal number of transplanted cells, immunosuppression regimens, and disease stage for transplantation [168].

Induced Pluripotent Stem Cells as a Therapeutic Option

The recent development of induced pluripotent stem (iPS) cells holds great promise as a potential reservoir of cells for the treatment of age-related macular degeneration and other disorders. Induced pluripotent stem cells were initially generated in 2006 by the Yamanaka group and the technology has had dramatic implications both from an ethical and scientific standpoint [169]. The technology is a significant advancement over prior technology, as it allows researchers to generate pluripotent cells for

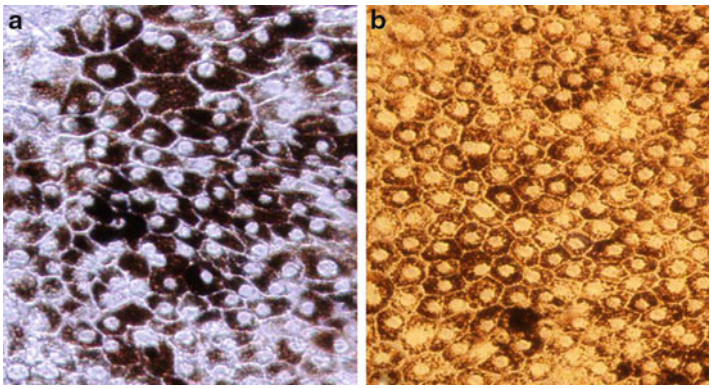

Fig. 1.11 Generation of RPE-like cells from human-induced pluripotent stem (iPS) cells. RPE derived from iPS grown on PA6 feeder cells (**a**, **b**). PA6 feeder cells exhibit stromal-derived inducing activity (SDIA), which promotes differentiation into RPE (Image courtesy of Stephen Tsang)

potential therapeutic use without the controversial use of embryos. These cells are generated by reprogramming adult somatic cells using transcriptional regulators such as *SOX2*, *OCT3/4*, and *Klf4* [169, 170]. These cells are then reprogrammed with similar potential as embryonic stem cells and are capable of differentiating into three germ layer cell types (mesoderm, ectoderm, and endoderm) [169, 171, 172]. These iPS cells hold great promise for the generation of RPE and photoreceptors for cell replacement therapy and create a new paradigm as a novel reservoir. Tsang et al. have generated RPE-like cells from human iPS (Fig. 1.11). Several other groups have used iPS technology to generate photoreceptors and then transplant these cells into animal models of retinal degeneration [173–175].

Induced pluripotent stem cells can also provide a platform to study disease through the use of patient-specific iPS cells. Through the generation of iPS cells from patients with specific diseases, models can be developed to express particular disease phenotypes which can then be used to understand pathophysiology of disease and determine the efficacy of therapeutic interventions [176]. These models can also be developed to help understand human inherited diseases given their clinical and genetic heterogeneity [177]. Cells derived from a particular patient can be used as a biological tool for drug discovery and toxicity testing of therapeutic agents, providing a new paradigm for personalized medicine [176].

Utilizing iPS cells as a tool for cell replacement therapy could also reduce the possibility of immune rejection given their autologous nature. Use of patient-specific iPS-derived RPE, generated from somatic cells of the potential transplant recipient with geographic atrophy, has one major and important theoretic advantage over other potential cell sources, namely, the avoidance of graft rejection. This is an important advantage, since long-term systemic immune suppression is poorly tolerated in elderly patients. Although the presence of anterior chamber-associated immune deviation (ACAID) confers some immune privilege in the subretinal space,

allogeneic RPE will undergo graft rejection after subretinal transplantation unless immune suppression is used [178, 179]. Use of patient-specific iPS may circumvent graft rejection, which is one of the major challenges to ensuring graft survival in the subretinal space.

Challenges remain to the successful use of iPS cells. Induced pluripotent cells derived from the affected patient contain the predisposing mutation that caused the disease. This can provide a unique disease model but the mutation may also impede the function of the transplanted cells. These stem cells may have to first be repaired by targeted gene therapy or other techniques prior to transplantation. Additional work is also needed to translate the advances of iPS cells into clinical trials to assess safety and efficacy. Better understanding of iPS cell technology and refining the methodology of their generation will have a significant impact on retinal degenerations and regenerative medicine.

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Chapter 2
Stem Cells in Oculofacial Plastic Surgery

Bryan J. Winn and Mary Whitman

Abstract In the field of oculofacial plastic surgery, stem cells are beginning to be used in reconstructive and aesthetic applications. Adult mesenchymal stem cells, specifically adipose-derived stem cells (ADSCs), with their abundant supply, ease of harvest, and ability to differentiate into fat, bone, cartilage, muscle, and blood vessels appear to be excellent progenitor cells for use in facial reconstruction. ADSCs secrete cytokines which can enhance their own survival and engraftment. In addition, ADSCs have been utilized clinically for tissue engineering of facial structures including bone, cartilage, and fat and have the potential for engineering other mesenchymal structures the tarsus. Stem cells may augment wound healing, especially in the case of chronic wounds, free grafts, and flaps and theoretically could improve surgical outcomes, especially in high-risk settings. Lastly, the paracrine effect of adult mesenchymal stem cells has the potential to mitigate, and in some instances reverse, the process of age and oxidative skin damage. Well-designed, prospective, quantitative human trials need to be conducted to bring stem cell technology into standard oculofacial plastic surgical practice.

Introduction

With the potential to regenerate any kind of tissue, stem cell technology offers possibilities in all areas of medicine. In the field of oculofacial plastic surgery, stem cells are beginning to be used in reconstructive and aesthetic applications.

Embryonic stem cells (ESCs) are pluripotent and thus able to generate any cell type. However, since they are derived from fertilized embryos, ESCs present ethical

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S.H. Tsang (ed.), *Stem Cell Biology and Regenerative Medicine in Ophthalmology*,
Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-5493-9_2,

challenges in their clinical application, as well as the possibility of rejection by the host immune system. Adult mesenchymal stem cells (MSCs), or stromal stem cells as they are sometimes called, have been isolated from the stromal compartments of several mesodermal tissues including bone marrow, muscle, perichondrium, and adipose tissue [1–5]. MSCs demonstrate vast proliferative capacity and multilineage potential including the ability to differentiate into bone, muscle, cartilage, and fat as well as certain non-mesodermal structures such as neurons and Schwann cells [6–10]. For plastic and reconstructive surgery, MSCs have obvious advantages over ESCs as they do not present any ethical issues, are autologously obtained thus eliminating the concerns about rejection, and can easily be harvested in large supply.

MSCs have been shown to migrate to the areas of injured, ischemic, or inflamed tissue, increase tissue angiogenesis, and help the repair of injured tissues through growth factor secretion (such as vascular endothelial growth factor and hepatocyte growth factor) and matrix remodeling [11–13]. While bone marrow-derived MSCs have been the primary source for therapeutic applications for the past 20 years, recent studies have demonstrated that MSCs can be harvested from adipose tissue via ex vivo expansion through serial passaging [6]. The abundant supply of adipose, often a by-product of common procedures such as liposuction, fat transfer, and blepharoplasty; the potential to differentiate into bone, muscle, cartilage, and possibly tarsus; and the functional characteristics of its MSCs make adipose-derived stem cells (ADSCs) attractive to the field of aesthetic and reconstructive oculofacial plastic surgery.

Adipose-Derived Stem Cells

Although Robdell first isolated a population of progenitor cells from rat adipose tissue in 1964, a third of a century would pass before ADSCs would be found to reside in lipoaspirate, a disposable by-product of liposuction, and recognized for their similarities to bone marrow-derived MSCs [6, 14]. In the process of liposuction, after a tumescent anesthetic solution is injected under the skin, a blunt cannula inserted through a small skin incision is used to aspirate the fat and tumescent solution. To harvest ADSCs, this lipoaspirate then undergoes collagenase digestion and centrifugation to isolate the stromal vascular fraction (SVF) pellet containing the ADSCs [11]. These ADSCs have been shown to easily differentiate into mesenchymal cells such as bone, fat, cartilage, and muscle and have the ability to undergo self-renewal [15, 16].

Adipose tissue contains up to 1 % ADSCs which is in stark contrast to the 0.001 % stem cell fraction found in bone marrow [17]. However, studies have demonstrated that not all fat depots are equal in terms of quality of associated ADSCs. ADSCs harvested from the superficial abdominal depot above Scarpa's layer have been shown to be more resistant to apoptosis than other subcutaneous depots including the arm, hip, and thigh regions [18, 19]. In addition, younger patients appear to have increased induction of their ADSCs than older patients [18]. Once harvested and isolated, ADSCs can be expanded in a monolayer tissue culture

to be used in clinical experiments and applications or cryopreserved for up to 6 months after harvest [15, 20].

Although there is great interest in the clinical applications of ADSCs, this field is still in its infancy. With the ability to be easily and safely obtained in large quantities from lipoaspirate, ADSCs are beginning to be of clinical interest to the oculofacial plastic surgeon in the areas of wound healing, tissue engineering, autologous fat grafting, and skin rejuvenation.

Orbital Stem Cells

While most adipose tissue in the human body is derived from mesenchyme, orbital fat and the fat surrounding the paratracheal region is uniquely of neural crest origin [21]. In 2009, Korn and colleagues isolated and characterized adult stem cells from human orbital fat excised during routine blepharoplasty [22]. Korn found that stem cells derived from orbital fat carried neuronal cell surface markers and could be induced to express neuronal and glial antigens in tissue culture. The authors postulate that these neural crest origin stem cells could have potential therapeutic uses in the treatment of retinal dystrophies, trabecular meshwork reconstruction, ganglion cell replacement, and ocular surface reconstruction.

Kang and colleagues reported using fat excised during cosmetic blepharoplasty to isolate stem cells with neural crest origin characteristics in order to treat diabetes. After harvest, these stem cells were cultured with nicotinamide, activin, and GLP-1 to allow for differentiation into insulin secreting cells. These cells were then transplanted into streptozotocin-treated immunocompetent type I diabetic mice. Kang found that in 50 % of the mice, hyperglycemia normalized and only human, not murine, insulin and c-peptide was found in the blood of the mice. At 2 months, these cells continued to function and there was no sign of rejection [23]. The authors suggest that the success of this xenogeneic transplant may be due to low levels of HLA class I and the absence of HLA-DR, HLA-DM, CD80, and CD86 molecules expressed on the surface of these stem cells. This technology holds promise as a possible cure for type I diabetes in humans.

Clinical Applications

Tissue Engineering and Grafting

In reconstructive plastic surgery, the principle of “replace like with like” is well respected and practiced whenever feasible. However, in periocular reconstruction, tissues the tarsus and conjunctiva are often in too limited a supply to be successfully used for grafting purposes without creating significant donor site morbidity. Autologous ear cartilage and hard palate have been used as substitutes for tarsus, and

buccal mucous membrane for conjunctiva. However, donor site morbidity and increased surgical time related to graft harvesting have led surgeons to search for alternative materials. Many surgeons have embraced human acellular dermis (Alloderm, Lifecell, Branchburg, NJ), or bioengineered materials cross-linked porcine dermal collagen (ENDURAGen, Stryker CMF, Newnan, GA) and synthetics such as tarSys (IOP Ophthalmics, Costa Mesa, CA) to substitute for tarsus [24–26]. Similarly, banked amniotic membrane is now often used instead of conjunctiva or buccal mucous membrane for socket reconstructions [27, 28]. However, each substitute has its limitations. The prospect of growing abundant, autogenous, adult stem cell-derived tissues such as tarsus, conjunctiva, bone, adipose, and skin to be used for reconstruction is extremely attractive to the oculofacial plastic surgery community.

In 1993, Langer and Vacanti outlined the three fundamental strategies of tissue engineering: isolated cells or cell substitutes, tissue-inducing substances, and cells placed on or within a matrix [29]. While these "pillars" remain today, scientists are beginning to realize the critical role that the interaction between each of these pillars plays in the bioengineering of larger tissues and organs.

As discussed earlier, ethical concerns limit the use of embryonic stem cells. However, adult mesenchymal stem cells, specifically ADSCs, with their abundant supply, ease of harvest, and ability to differentiate into fat, bone, cartilage, muscle, and blood vessels appear to be excellent progenitor cells for use in facial reconstruction. ADSCs, however, require highly regulated and critically timed signals, in the form of biomolecules and growth factors, to allow for differentiation into specific surgically useful tissues. For instance, fibroblast growth factor-2 (FGF-2) promotes cartilage differentiation but inhibits bone differentiation [30]. Culturing ADSCs in low-oxygen-tension conditions may enhance proliferation, differentiation, and growth factor proliferation [31]. In addition, the ADSCs themselves are able to secrete angiogenic biomolecules such as hepatocyte growth factor, vascular endothelial growth factor (VEGF), stromal-derived factor-1 alpha, and granulocyte/macrophage colony-stimulating factor which are likely critical for survival and engraftment of stem cell-derived tissues [15].

Scaffolds or matrixes create an extracellular environment for the ADSCs, providing biologic structural cues, protection and a means by which the primed ADSCs can be introduced into the body of the recipient [32]. Several scaffold matrix materials with various chemical compositions, three-dimensional structures and degrees of mechanical stability have shown promise in supporting ADSCs. These include hyaluronic acid and collagen sponges, placental decellular matrix (PDM), silk fibroin-chitosan scaffold, and injectable collagen microbeads and poly (lactic-co-glycolic acid) spheres [15].

Although stem cell-oriented tissue engineering is in its infancy in terms of clinical applications, a small number of cases have been reported describing reconstructions utilizing cell culture and stem cell technology that could be applied to periocular reconstruction.

Cartilage

Yanaga and colleagues reported using cultured autologous auricular chondrocytes for nasal augmentation in 75 patients [33]. In this study, harvested chondrocytes from the auricular concha were cultured on a gelatinous chondroid matrix and then injected into the subcutaneous nasal dorsum. The matrix changed from a soft gel to rigid neocartilage within 2–3 weeks of implantation and produced good long-term results [33]. Yanaga also reported using a 2-stage technique for the treatment of microtia in 4 children [34]. Chondrocytes were harvested from the auricular remnant and cultured in a multilayered fashion as in the prior study. This chondroid matrix was then injected subcutaneously into the abdomen to allow for growth into a large block of cartilage with a neoperichondrium. At 6 months, the graft was harvested from the abdomen, sculpted into the shape of auricular cartilage, and implanted subcutaneously in the area of the microtia to create the form of a new ear. At 2–5 years follow-up, the graft retained good shape without evidence of reabsorption. This technique allowed for minimal donor site morbidity and the generation of enough graft material to create a complete auricular cartilage structure.

Bone

In 2001, Quarto and colleagues reported the first clinical application of autologous adult stem cells in treating large long bone defects in 3 patients [35]. Stem cells were isolated from bone marrow, expanded ex vivo, placed on hydroxyapatite scaffolds tailored to fit the size of the specific bony defect, and implanted. External fixation was removed at 6–13 months and at 15–27 months there were no issues with the implants and all patients recovered limb function. This technique greatly reduced recovery time and morbidity compared with traditional noncellular implants. Warnke and colleagues later reported applying stem cell technology to reconstruct a 7 cm mandible defect after subtotal mandibulectomy [36]. For this technique, three-dimensional computed tomography was used to design a titanium mesh cage that would be a virtual replacement for the missing bone. The cage was then filled with bone mineral blocks and injected with human bone morphogenic protein 7 (BMP7) and the patient's non-processed bone marrow. This combination of scaffold, tissue-inducing substance, and stem cells was then implanted in a pocket created in the patient's latissimus dorsi muscle and transplanted as a free bone-muscle flap into the mandibular defect 7 weeks later. The patient had improved mastication and subjective aesthetic appearance following the procedure [36].

Adipose

Autologus adipose has been traditionally used for a number of aesthetic and reconstructive indications, including hemifacial atrophy; soft tissue defects following infection, trauma or radiation; facial augmentation and facial rejuvenation

[11, 37–39]. Unfortunately, autologous fat grafts have survival rates between 25 % and 60 % making their use clinically somewhat unpredictable; often multiple grafting sessions are required to achieve satisfactory results [13]. Cell-assisted lipotransfer (CAL) is a technique by which adipose-derived stem cells (ADSCs) are used to augment standard lipoinjection. Lipoaspirate, typically harvested from the abdomen, is divided in half. One half undergoes collagenase digestion and centrifugation to isolate the stromal vascular fraction (SVF) as described earlier. The SVF is then added back to remaining lipoaspirate to create ADSC-rich fat, which can then be injected in the manner of traditional lipotransfer. Compared with traditional lipotransfer, CAL has been shown to have 35 % improved graft survival with microvasculature detected more prominently in the outer layers [40].

Sterodimas and colleagues reported a prospective, randomized, non-blinded, interventional study of 20 patients with congenital or acquired facial tissue defects who were treated with either traditional lipotransfer or CAL [11]. In the traditional lipotransfer group, 30 % achieved aesthetically acceptable results after the initial treatment with the remaining 70 % requiring one or more additional treatments. In the CAL group, 100 % of the patients required only a single treatment. While those in the CAL group had higher initial patient satisfaction scores, at 18 months there was no difference between groups. There were no complications in either group.

In a separate report, the same authors describe a case of 19-year-old patient with Parry–Romberg syndrome and progressive hemifacial atrophy who was treated with 90 cc of cell-assisted lipotransfer. At 1 year, the patient was satisfied with the aesthetic result and did not require further treatment [15].

It is unclear the precise role ADSC plays in CAL. Preadipocytes and ADSCs are thought to be more resistant than mature adipocytes to the trauma of graft harvest and postimplantation ischemia [11, 41–43]. In fact, ADSCs have been demonstrated to increase proliferation in response to hypoxia [44]. In addition, ADSCs are known to secrete VEGF and hepatocyte growth factor both of which may contribute to neoangiogenesis following implantation [11]. ADSCs may also, themselves, act as vascular endothelial progenitor cells [45]. Hence, it follows logically that grafts with higher concentrations of ACSCs may have improved and less variable survival.

Wound Therapy

Normal wound healing occurs in four distinct but overlapping phases: hemostasis, inflammation, proliferation, and remodeling. In the abnormal, chronic wound, ischemia and bacterial overgrowth lead to a relentless cycle of inflammation and tissue injury. While the oculofacial plastic surgeon rarely encounters venous ulcers, pressure ulcers and diabetic ulcers which comprise the majority of non-healing wounds, poor wound healing can present in facial burns, advancement flaps and free grafts, especially in the setting of previously irradiated or scarred tissue, infection, smoking, and advanced age [16, 46].

As discussed earlier, mesenchymal stem cells have the ability to differentiate into multiple cell types and release pro-angiogenic cytokines which likely benefit tissues undergoing wound healing. In addition, the low oxygen tension of chronic wounds, flaps and free grafts may actually further stimulate mesenchymal stem cells to proliferate and release growth factors [15, 31].

Simman and colleagues, in a prospective, interventional murine experiment, found that "priming" the donor site of a skin flap with subcutaneously injected bone marrow-derived stem cells and angiogenic factors 1 week prior to flap elevation significantly improved flap survival compared to priming with only control medium, angiogenic factors alone, or stem cells alone. Interestingly, the authors found that the same introduction of angiogenic factors and stem cells did not significantly improve flap survival when injected at the time of flap elevation [47].

With the ease of harvest and abundant supply of adipose-derived stem cells (ADSCs), investigators have recently focused on the therapeutic uses of ADSCs for wound healing. Kim and colleagues found that ADSCs promote dermal fibroblast proliferation and significantly accelerate the rate of wound closure without the formation of hypertrophic scar [48]. In addition, Ebrahimian and colleagues determined that ADSCs can differentiate into keratinocytes and produce angiogenic growth factors in both normal and irradiated tissues [49]. Nambu showed that the negative effect of diabetes mellitus on wound healing could be counteracted by the introduction of ADSCs to the wound [50]. Furthermore, Altman found that human acellular dermal matrix seeded with ADSCs differentiated into vascular endothelial, fibroblastic, and epidermal epithelial cells after in vivo engraftment and significantly improved wound healing [51].

The application of stem cell technology for wound healing was recently described in a cohort of human subjects. Rigotti and colleagues demonstrated significant improvements in radiation-induced wounds of the chest wall and supraclavicular region with the injection of ADSC-rich purified lipoaspirate. In this study 95 % of the 20 patients showed improvement of their wounds following one or more injections [52].

From these studies, it follows that stem cells have the potential to improve wound healing and could prove clinically useful, especially in high-risk settings. Further human studies need to be performed to determine the optimal methods and surgical timing for stem cell-assisted wound therapy.

Skin Rejuvenation

During normal aging the skin becomes less elastic, irregularly pigmented, and thinner. Both genetic predisposition and environmental factors such as smoking and ultraviolet light exposure have been shown to contribute to age-related skin damage. Mesenchymal stem cells are thought to counteract the appearance of skin aging not only via direct cell-to-cell interactions but also though paracrine effects of the various secreted growth factors and cytokines [53]. For example, ADSC culture medium (ADSC-CM) containing secreted cytokines and growth factors from

ADSCs has been shown to induce dermal fibroblast migration and enhance type I collagen secretion [48].

ADSCs may have a significant role in protecting skin from damage due to oxidative stress. In animal models of oxidative skin injury, ADSC-CM protected dermal fibroblasts against *t*-butyl hydroperoxide free radical damage and inhibited apoptotic cell death induced by reactive oxygen species [53]. In addition, ADSC-CM has been shown to inhibit melanoma B-16 cells by arresting them in the G1 phase, thus delaying the cancer progression [53].

Ultraviolet radiation is at least partly responsible for skin pigmentation and fine wrinkling. Kim and colleagues created a murine model of UV light-induced wrinkles and examined the effects of ADSCs. Wrinkling was significantly lessened while dermal thickness and collagen content in the dermis was increased after subcutaneous injection of ADSCs [53]. Furthermore, ADSC-CM increased the production of type I collagen and decreased the level of matrix metalloprotease 1 (MMP 1) in fibroblasts which may contribute to the thickened dermis [54]. The paracrine effects of ADSCs also appear to alter pigmentation. ADSC-CM was found to inhibit melanin synthesis and tyrosinase in melanoma B16 cells and therefore may be useful to treat the dyspigmentation associated with photodamage [55].

Park and colleagues report a case of a human subject receiving injections of purified autologous lipoaspirate containing approximately 20–30 % ADSCs to treat photoaged periorbital skin [56]. The patient received two injections 2 weeks apart and at 2 months the texture of the patient's skin and fine wrinkles were subjectively improved. In addition, the dermal thickness over the area of treatment had increased by over 10 % (2.054 mm vs. 2.317 mm) after the treatment.

These studies suggest that stem cells and their paracrine effects may significantly improve age-related and oxidative skin damage by inducing fibroblast migration, increasing collagen production, protecting against free radical damage, inhibiting matrix metalloproteases, and inhibiting dyspigmentation. Further human studies are necessary to bring this technology to clinical practice.

Conclusions

In the field of oculofacial plastic surgery, the application of stem cell technology has the potential to improve both aesthetic and reconstructive outcomes. Autologous adipose-derived stem cells have the potential to allow for tissue engineering of vital periocular structures, improve wound healing and the success of tissue grafts and flaps, and augment the ability to aesthetically treat the aging face. Additional well-designed, prospective, quantitative human trials need to be conducted to bring this technology into standard clinical practice.

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Chapter 3
The Current Status of Corneal Limbal Stem Cell Transplantation in Humans

Roy S. Chuck, Alexandra A. Herzlich, and Philip Niles

Abstract The cornea provides an accessible source of adult stem cells for cell-based therapies. Corneal stem cells have been discovered in the three primary strata—epithelium, stroma, and endothelium—of the cornea. Limbal epithelial stem cells are found on the surface and are able to differentiate into transient amplifying cells, which can regenerate epithelial tissues. Limbal stem cell deficiencies can result in epithelial defects, ulceration, corneal vascularization, chronic inflammation, scarring, and conjunctivalization of the cornea. Stromal stem cells share many properties with bone marrow-derived stem cells. Though stromal stem cell research is in the early stages, these cells may one day provide bio-prosthetic stromal material. Endothelial stem cells may be of particular importance due to endothelial cell damage during common surgeries and degenerative diseases. Current stem cell therapies focus on regeneration of the corneal surface by replacing limbal epithelial stem cells with corneal-derived cells, other adult stem cells or embryonic stem cells. Advances in cell culturing will hopefully soon be translated from bench to bedside to help in the treatment of severe ocular surface disease.

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S.H. Tsang (ed.), *Stem Cell Biology and Regenerative Medicine in Ophthalmology*,
Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-5493-9_3,

Introduction

The use of embryological stem cells has been the center of ethical and practical dilemmas in the scientific community. Luckily, the cornea offers the possibility of using easily accessible adult stem cells for cell-based therapies for different ocular diseases.

Corneal Anatomy

The cornea is the anterior, transparent, avascular layer of the eye. The anterior surface is covered by a layer of non-keratinized stratified squamous epithelium five to six cells thick. Originally derived from surface ectoderm, these cells are joined together by tight junctions that prevent tear fluid from entering the stroma. This layer continues to the edge of the conjunctival epithelium, forming the limbus. The epithelium and tear film form the smooth optical surface with high refractive power that directs light to the retina. The second layer, Bowman's layer, is the avascular condensate of the outer layer of the stroma. The corneal stroma, the third layer, consists of cells originally derived from neural crest. The densely packed collagen type I and V fibers are arranged in parallel bundles, which are then packed in stacked parallel lamellae creating an organized, transparent, and strong unit. Keratocytes are the main cell type of the stroma and help maintain the extracellular matrix by regulating matrix metalloproteases. The next layer, Descemet's membrane, is the thick basement membrane secreted after birth by the last layer, the corneal endothelium. Also derived from neural crest, the endothelium is a layer of closely interdigitated hexagonal cells critical for the maintenance of corneal transparency through use of its ATPase-dependent metabolic pump [1–4] (see Fig. 3.1a, b).

At the limbal zone, where the corneal meets the conjunctival epithelium, the corneal structure changes. The epithelium thickens and forms epithelial pegs made up of 11–12 cell layers instead of 5. Bowman's and Descemet's layers are missing and the undulated epithelium basement membrane lies above the limbal stroma. The collagen bundles become less organized and cells are abundant and fibroblast-like. The endothelial cells here are larger and flatter than in the central cornea. The bulbar conjunctiva becomes more adherent to Tenon's capsule; and these tissues blend to make up radiating ridges called palisades of Vogt. This area is thought to contain corneal epithelial stem cells [1–3] (see Fig. 3.1c). Although it has been hard to definitively identify limbal epithelial stem cells, a breakthrough came with the identification of transient amplifying cells (TACs). These cells are a differentiated and rapidly proliferating epithelial cell type and are found both in the peripheral and central cornea, but not in the limbus, providing further evidence for the existence of stem cells specifically in the limbus [5].

Corneal endothelial cells (CEC) originate from the neural crest [6] and have a monolayer arrangement. The endothelial cell density increases from the center to the periphery [7]. CECs are connected by focal tight junctions that create a

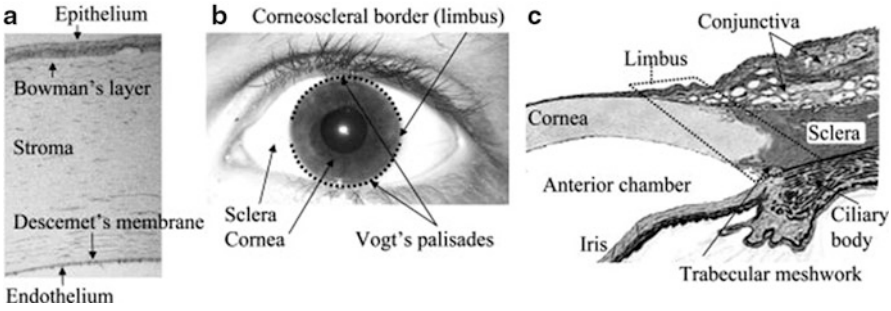

Fig. 3.1 (**a**) Histology of the human cornea stained with hematoxylin and eosin (H&E). (**b**) Corneoscleral border. (**c**) Limbal junction. Reprinted with permission from Takacs et al. [3]

semi-permeable barrier, which allows water and nutrients from the aqueous humor to enter the stroma. CECs also contain ionic pumps that expel water from the stroma [8]. This fluid balance maintains corneal clarity.

Definition of Corneal Stem Cells

Adult stem cell populations are found in most tissues. These are cells that are able to maintain and regenerate a given tissue for a long time. They are characterized by three main properties. First, the cells must be capable of self-renewal: one of the dividing cells must always remain a stem cell. Second, the cells must remain in an undifferentiated state but maintain the potential for differentiation. Finally, the cells must remain in a growth-arrested state, supported by a microenvironment that provides external factors necessary for maintaining stem cell properties, until they are required to differentiate [9].

Corneal epithelial stem cells can be stimulated to divide and differentiate into transient amplifying cells. They continue to divide into terminally differentiated corneal epithelial cells and migrate centripetally for the continued replacement and regeneration of tissues following injury. This maintains a steady-state population of healthy cells.

Corneal stromal cells are stromal keratocytes, of neural cell origin. They help form an interconnected network of stromal lamellae and are responsible for the deposition of stromal extracellular matrix and for maintenance of corneal transparency. Stromal stem cells have the ability to differentiate from keratocytes into fibroblastic cells in the face of irritation such as wounds, infections, and corneal pathologies. Keratocyte-derived fibroblasts secrete fibrotic matrix components that disrupt corneal transparency but allow for wound healing and strength [10, 11].

Corneal endothelial stem cells (CESC) are particularly important because corneal endothelial cells have decreased proliferative capacity, particularly after the age of 20 years [12]. CESCs are believed to be located in the transition zone of the posterior limbus and to be active after corneal wounding [13]. However, much more research is required to understand how CESCs respond to endothelial damage.

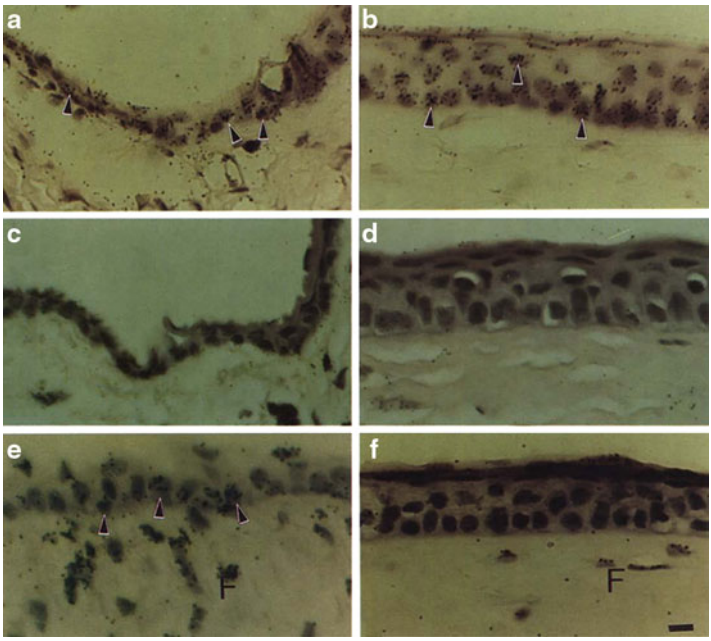

Fig. 3.2 Epithelial stem cells. Identification of label-retaining cells (LRC) in limbal epithelium. Autoradiograms demonstrate the labeling pattern of limbal (**a**, **c**, **e**) and central corneal (**b**, **d**, **f**) epithelia after long-term labeling under various conditions. After 2 weeks of continuous 3H-TdR labeling (**a**, **b**), nuclei of almost all corneal and limbal epithelial cells contain silver grains (*arrowheads*). Most of these labeled cells disappeared from both limbal (**c**) and corneal (**d**) epithelia after a 4-week chase. Wounding during the continuous labeling followed by a similar 4-week chase revealed a population of label-retaining limbal basal cells (*arrowheads*) in (**e**). No LRCs were observed in the corneal epithelium (**f**). Some fibroblasts (F) in limbal (**e**) and corneal stroma (**f**) also retain labels: these cells were probably stimulated to incorporate 3H-TdR during wounding and then returned to a normal state during chasing. Calibration bar (**a**–**f**) = 8 pm. Reprinted with permission from Cotsarelis et al. [16]

Limbal Epithelial Stem Cells

The corneal epithelium is a continuously regenerating surface tissue. In 1971, Davanger and Evenson proposed that the limbus could be the reservoir of new epithelial cells when they first characterized the central migration of cells from the limbus [14]. Further studies described the distance of migration in a mouse model to be 94 ± 14 μm in 7 days [15]. In support of the capability of self-renewal, Cotsarelis' group noted that a population of slow cycling cells labeled with tritiated thymidine retained an easily detectable amount of thymidine while the amount of label per cell quickly decays in more frequently dividing cells [16] (see Fig. 3.2). From the percentage of thymidine retaining cells in the limbal zone, it has been concluded that stem cells may represent less than 10 % of the total limbal basal cell population [17]. Dua and Forrester were able to demonstrate that limbal cells were involved in wound healing, proving that these limbal cells met all criteria of stem

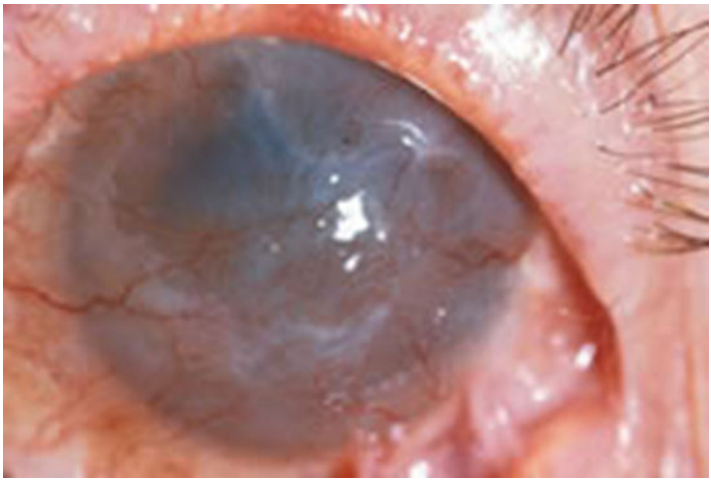

Fig. 3.3 Limbal stem cell deficiency in a 25-year-old patient with Steven–Johnson syndrome

cells [18]. Furthermore, central cornea cells are biochemically different than limbal cells based on keratin expression providing further evidence that they are a distinct population of cells [19].

Once limbal stem cells were identified, many groups were able to culture and further identify stem cell features of these cells. Pelligrini's group was able to demonstrate the expression of alpha enolase, alpha-6 integrin, low CD71, and no connexin 43 [20]. They also showed an increased expression of nuclear p63 by clonal analysis [21].

Since these advances were made, the location and expression of limbal epithelial stem cells (LESCs) as well as their migration have become a subject of debate. As recently as 2010, Majo et al. demonstrated that despite extensive limbal cell damage, corneal integrity and clarity were maintained for 4 months, claiming that oligopotent stem cells exist throughout the cornea surface and that the limbal, central, and conjunctival epithelium all share similar properties [22]. In addition, others have found that despite clinically evident total limbal cell deficiency, corneal epithelial regeneration is maintained, providing further evidence that corneal epithelial stem cells may be present throughout the cornea epithelium [23].

Limbal stem cells are now accepted to be of small size and have a high nucleus to cytoplasm ratio. They are normally quiescent, which is thought to reduce the possibility of DNA damage. Limbal stem cells retain the possibility of differentiating into many different and distant cell types including hair follicles. Conversely, epidermal stem cells are capable of differentiating into corneal epithelial cells and repairing a damaged corneal surface in the setting of total limbal stem cell deficiency, as shown in a goat model [24].

Limbal stem cell deficiency (LSCD) is characterized by persistent or recurrent epithelial defects, ulceration, corneal vascularization, chronic inflammation, scarring, and conjunctivalization of the cornea with loss of clear demarcation of the cornea and conjunctival epithelium at the limbus [25] (see Fig. 3.3). Causes of LSCD are both

hereditary and acquired. Hereditary causes include aniridia and keratitis associated with multiple endocrine deficiency (MEN), in which stem cells may be congenitally absent or dysfunctional. Acquired causes are much more common in clinical practice and include Steven–Johnson syndrome (SJS), chemical injuries, ocular cicatricial pemphigoid (OCP), contact lens-induced trauma, neurotrophic degenerations, and peripheral ulcerative keratitis.

The chronic instability of the corneal epithelium caused by LSCD leads to persistent corneal epithelial breakdown, superficial corneal vascularization, chronic discomfort, and impaired vision. This may lead to progressive melting of the cornea, and can cause subsequent perforation. This sequence has been demonstrated in animal models in which removal of the limbus resulted in insufficient re-epithelialization and conjunctival invasion of corneal surface [26]. Furthermore, during wound healing it has been found that there is an increase in the number of cells in the limbal area [27].

Stromal Stem Cells

The main cell type of the stroma is the keratocyte. In response to wound healing, keratocytes proliferate and become fibroblastic. This enables them to secrete fibrotic matrix components creating a more structurally stable interface at the expense of corneal clarity. Early work with keratocytes aimed to elucidate environmental influences on morphological changes; however, multiple groups found evidence of stromal stem cells or, at very least, stromal progenitor cells in both human cell lines and animal models. These progenitor cells are differentiated from primary keratocytes in a few key ways. They have the ability to form spontaneous aggregations of attachment independent spheroids. These spheroids are solid balls of viable cells that express high levels of keratocyte-specific genes. They express a number of genes known to be up-regulated in multiple stem cells types, such as *Notch-1, Bmi1, SCF, FHLIM1, ABCG2, CD73, CD90*, and *CD166*. They also express high levels of the genes *PAX6, Six3, Six2*, all of which are associated with eye development [28, 29]. Further support for these stromal stem cells comes from work where the progenitor stromal cells of C57BL6/J mice were cultured and conditioned to differentiate into either stromal fibroblasts or a-SMA-positive myofibroblasts, or to maintain primary keratocyte properties if cultured in a different serum media [30] (see Fig. 3.4).

Though the field of stromal stem cell research is in its earliest stages, it provides an optimistic outlook for providing bio-prosthetic stromal material. The stromal progenitor cells have been shown to exhibit multipotent differentiation potential. Perhaps most importantly, corneal stromal stem cells have been shown to be capable of secreting an organized tissue-like extracellular matrix resembling that found in the corneal stroma. This matrix can be populated with keratocyte-like cells, with significant biomedical engineering implications [31].

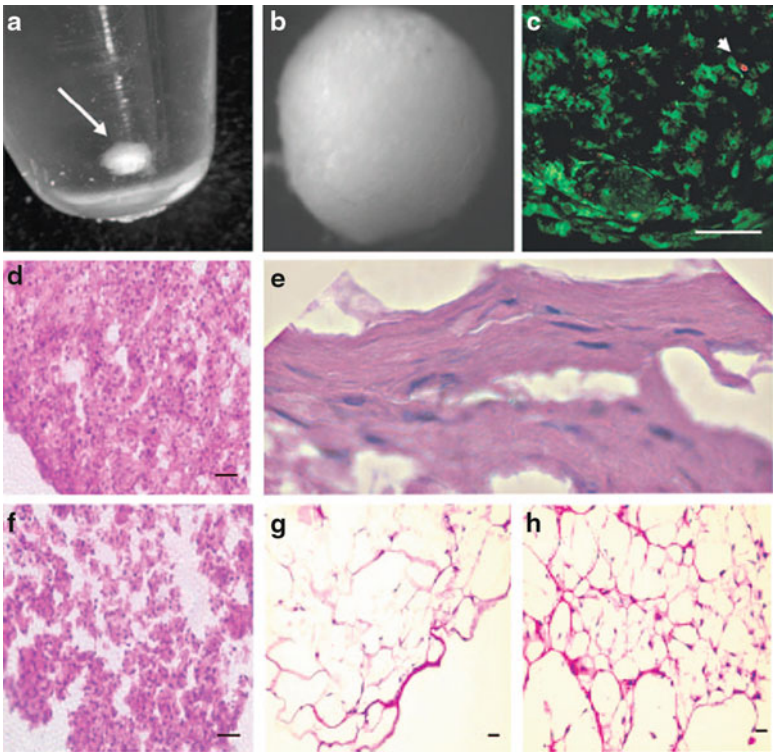

Fig. 3.4 Stromal stem cells. Three-dimensional cultures of human corneal stromal stem cells (hCSSC). (**a**) Formation of a free-floating pellet after centrifugation of 2×10^5 hCSSC in a 15-mL conical polystyrene tube (*arrow*). (**b**) Pellet has formed a smooth sphere after 1 week of culture. (**c**) Identification of viable cells after staining with calcein AM (*green*) and dead cells using propidium iodide (*red*, *arrowhead*) after 3 weeks of culture. (**d**) H&E staining of hCSSC cultured 3 weeks as a pellet. (**e**) Flattened cells near the periphery of an hCSSC pellet. (**f**) H&E of a pellet formed by fibroblasts cultured for 3 weeks. (**g**) Stained section of hCSSC cultured in a fibrin gel for 3 weeks. (**h**) Fibroblasts cultured in fibrin gel. Scale bars, 50 μm. Reprinted with permission from Du et al. [31]

Endothelial Stem Cells

In 2005, Whitehart first demonstrated the existence of corneal endothelial stems cells (CESC). His group showed increased telomerase activity and incorporation of bromodeoxyuridine into cellular DNA after endothelial damage, which is suggestive of cellular division [32]. This work was expanded upon by McGowan in 2007, who found the stem cell markers nestin, alkaline phosphatase, Oct-3/4, Pax-6, Sox-2 and Wnt-1 in addition to telomerase in the trabecular meshwork and the transition zone of the posterior limbus, with several of these markers appearing in the endothelial periphery only after corneal wounding [13]. These studies formed the foundation for demonstrating the existence of corneal endothelial stem cells (see Fig. 3.5).

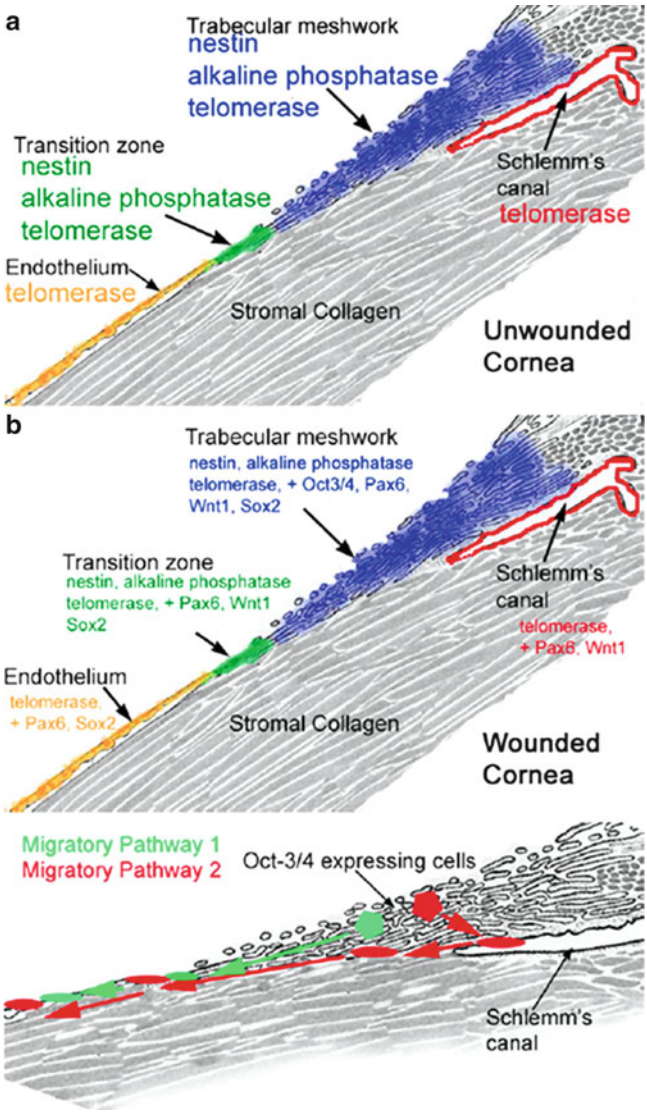

Fig. 3.5 Endothelial stem cells. Reprinted with permission from McGowann et al. [13]

Corneal endothelial cells in general have a comparatively reduced ability to divide [12], which has prompted research into the factors governing CESC division. Though the governing mechanisms of CESC proliferation are not yet fully understood, the most studied inhibiting factor of CESC proliferation is transforming growth factor-beta2 (TGF-beta2), which is present in the aqueous humor [33].

In multiple animal studies, TGF-beta2 has been shown to modulate endothelial wound healing. The endogenous presence of TGF-beta2 has been shown to be necessary for growth, while its exogenous addition may inhibit division [34, 35]. Regulators of CESC division will require further investigation to obtain a more complete understanding.

CESCs are of particular importance since the cells of the corneal endothelium lose their proliferative capacity with age, and may lose all proliferative capacity by the age of 20 years old [12]. Due to endothelial cells' decreased ability to proliferate, damage will often lead to a decreased endothelial cell density. Additionally, endothelial cells are susceptible to damage during common surgeries, such as cataract removal, and in the course of degenerative and dystrophic diseases. Though CESCs seem to respond to endothelial damage [13], the endothelium's primary response is endothelial flattening and expansion by approximately 25 % in order to cover the damaged area [36]. This change in cellular form may compromise the ability of the endothelial layer to retain its barrier function, making research and understanding of CESC proliferation and methods of transplantation [37] to be of particular therapeutic importance.

Current Treatments of Corneal Stem Cell Deficiencies

The potential for regeneration of the corneal epithelial surface has stimulated the creation of numerous techniques to replace limbal stem cells. These can be classified as one of the following procedures: conjunctival autograft (CAU), conjunctival allograft (CAL), conjunctival limbal autograft (CLAU), cadaveric conjunctival limbal allograft (c-CLAL), living related conjunctival limbal allograft (lr-CLAL), or keratolimbal allograft (KLAL).

In 1977, Thoft first described epithelial transplantation for severe ocular surface disease when he reported conjunctival transplantation for monocular chemical bums. By using the fellow eye he was able to avoid a major problem facing the allograft procedure, namely, immunologic rejection. Next Kenyon and Tseng modified the above procedure, naming it the conjunctival limbal autograft (CLAU) in 1989 [38]. This technique takes grafts of bulbar conjunctiva that extended approximately 0.5 mm onto the clear cornea centrally, thus containing limbal corneal cells. These techniques are now widely used by many corneal surgeons. However, the use of the technique is limited to cases where the donor eye is healthy, which subsequently puts it at risk for stem cell deficiency itself [39]. Another drawback includes the inability to use these techniques for ocular surface disease patients with the greatest need, namely, those affected severely bilaterally. A further modification came when Kenyon and Rapoza described a technique they called limbal allograft transplantation, giving rise to the lr-CLAL, in which they transplanted limbal tissue with a conjunctival carrier from a living-related donor. This technique was similar to the

previously described technique of CLAU, except that the donor tissue was obtained from a living relative as opposed to the fellow eye. Postoperative management included topical corticosteroids [39, 40–42].

More recently, the procedures have included the use of trephines to harvest, as well as sutures and/or glue to secure, the grafts. Allografts are important in treating patients with severe bilateral surface disease; however, they do require lifelong systemic immunotherapy [43–46]. Kim et al. showed that receiving systemic immunosuppression increases the success rate of the transplantation by 58 % over the use of topical immunosuppression alone [47]. Overall the success rate of limbal stem cell transplants at 10 years is up to 75 % in some trials [48].

The next modification of limbal stem cells involved amniotic membranes. First used in the 1940s, amniotic membrane [49], the inner most layer of placenta, has been shown to facilitate the proliferation, differentiation, and maintenance of epithelial cells [50]. Because of its properties it has been used as an alternative to conjunctival autografts for conjunctival reconstruction in pterygium and conjunctival neoplasia removal, as well as scars and symblepharon [51–52]. It has also been shown to promote ocular surface reconstruction in both the chronic and acute phase of limbal stem cell disruption by helping heal epithelial defect, improving limbal stem-cell function, and providing symptompatic pain relief [51–52]. Because of its success in epithelial healing and its action as a barrier against conjunctival invasion of the cornea, it has been proven effective in treatment of partial limbal stem cell deficiency as well as in combination with limbal autografts [53–54] to further promote healing of conjunctival and corneal defects.

Ex vivo stem cell expansion is the next step in the evolution of stem cell transplants. This involves transplanting ex vivo expanded autologous limbal stem cells from the fellow eyes of patients with unilateral LSCD. Pelligrini et al. described it in patients with unilateral alkaline injury; her group was able to use a small limbal biopsy of 2 $\times$ 2 mm from the patient's own healthy eye [47]. The cells were cultivated on either amniotic membrane or fibrin-based substrates. The composite graft was then transplanted to the recipient eye. The smaller biopsy graft minimizes potential damage to the healthy contralateral eye. The cultivated cells can also be cryopreserved and used in the event of repeat grafts. Similar results have been demonstrated by other groups as well [42, 46, 55–59]. Though long-term results and safety have yet to be determined, one study demonstrated cultivated limbal stem cell graft success at 10 years in more than 75 % of patients treated for ocular burns causing LSCD [59]. Another group showed that after allograft limbal stem cell transplants, the limbal cells seemed to be in a state of hyperproliferation, only demonstrating host DNA 9 months status post-transplant, suggesting that the transplant maybe a trigger for host cells to populate the ocular surface [60]. Griffith et al. have gone yet a step further by virally immortalizing corneal epithelial and endothelial stem cells to maintain their proliferative potential [61]. Clinical applications are still unclear.

Application of Non-ocular Adult and Embryonic Stem Cells for Treatment of Ocular Surface Disease

Searching for a source of novel cells that are similar to corneal cells, many groups have succeeded in finding other ways of further developing ocular surface reconstruction. Using the embryological similarities in lineage between corneal epithelium and epidermis, Yang was able to reconstruct damaged corneal surfaces using epidermal cells in goat models [24]. Monteiro et al. found similarities between corneal limbal stem cells and human immature dental pulp stem cells (hIDPSCs) providing another potential source for surface reconstruction [62]. Nasal mucosa was also found to be a very effective substitute for achieving ocular surface reconstruction in cases of severe OSD [63]. Even murine vibrissae hair follicle bulge-derived stem cells were reported to have therapeutic potential as an autologous stem cell source [64].

Also, and possibly most promising, there is the advent of oral mucosal epithelium as a source of cells to treat bilateral LSCD. There is no need for immunosuppression since the graft is autologous. Additionally, it is thought that oral mucosa is at an earlier stage of differentiation than corneal keratinocytes and can thus more easily proliferate. Finally, these cells express keratin 3, which is not expressed by epidermis, suggesting greater similarity to corneal epithelium [65]. Further decreasing the risk of rejection, transmission of infection, or the need for long-term corticosteroid and immunosuppressive therapy, Ang's group has investigated using autologous serum to cultivate oral epithelial cells, which further decreases the risk of rejection, transmission of infection, or the need for long-term corticosteroid and immunosuppressive therapy [66]. This group also found that the oral mucosa could be cultured on both amniotic membranes and in carrier-free cell sheets. Different groups have been treating severe ocular surface disease with cultivated oral mucosal epithelial transplantation since 2002 and have found sustained corneal epithelium reconstruction in these patients [65, 67].

Pluripotent human embryonic stem cells have recently been cultured in vitro. In theory, differentiation of these embryonic stem cells into a corneal epithelial lineage could be achieved by replication of the limbal stem cell environment. First attempted in 2004, in vitro differentiation of embryonic cells into cells that behave like corneal epithelium was demonstrated in a mouse model. More recently, flow cytometry and real-time PCR results confirm the similarities between the embryonically derived corneal cells and corneal epithelial cells. However, further studies are underway to overcome problems associated with functionality, immune rejection, and ethical concerns [68].

As described above, corneal cells, limbal epithelial, stromal and endothelial corneal, all play important roles in the maintenance of a healthy cornea. Much research is currently underway to better delineate the exact nature of these cells and the full clinical applications of regeneration therapies.

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Chapter 4
Lens Differentiation from Embryonic Stem (ES) and Induced Pluripotent Stem (iPS) Cells

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Abstract The formation of lens progenitor cells and differentiated lens tissue in cell culture conditions presents a number of experimental challenges, even though lens lineage formation and lens fiber cell differentiation are among the best characterized model systems at both genetic and molecular levels. Lens differentiation from ES cells in vitro appears to be a feasible goal. This chapter describes the significance of using ES and iPS cells for better understanding of embryonic lens development and formation of congenital cataracts. A discussion of how iPS cells can help studies of age-related cataract is also included. The chapter summarizes the current data on lentoid body formation from human and primate ES cells, and the molecular basis of directed differentiation of human ES cells into lens progenitor cells and lentoid bodies. Finally, current gaps in lens research and future directions to address these problems are discussed.

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S.H. Tsang (ed.), *Stem Cell Biology and Regenerative Medicine in Ophthalmology*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-5493-9_4,

Introduction

The central premise of embryonic stem (ES) cell biology is an unlimited potential of ES cells to form every cell type of the whole organism [33, 42, 91, 120]. The potential is fulfilled during ontogenesis. The major question is if it is possible to differentiate ES cells into all transient (embryonic germ cell layers and common cell progenitors) and terminally differentiated cell types in vitro. A large body of work using mostly human and mouse ES cells conducted during the last decade has shown that it is a generally feasible goal with major implications for our understanding of embryonic development; modeling of human disease and treatment of a wide range of diseases that require cell-based therapeutics [50].

The human eye is an excellent organ for in vitro studies of its organogenesis, modeling of human eye diseases through the generation of disease-specific-induced pluripotent stem (iPS) cells via nuclear reprogramming [121], and for cell replacement and paracrine rescue therapies [66]. To harness the power of ES- and iPS-cell-based ideas of treating human eye diseases, the essential first step is to develop procedures to form ocular cells and tissues using in vitro conditions. The main challenge for this research originates from our limited knowledge of cell fate specification processes that occur normally in a three-dimensional (3-D) context in developing embryos and what specific cell culture conditions may favor simultaneous formation of multiple cell types that might both positively and negatively influence the development of the desired cell type. While the cells can achieve the desired cell type, their terminal differentiation into a status comparable with tissues generated during ontogenesis often requires additional conditions that have to be determined empirically.

The formation of lens progenitor cells and differentiated lens in cell culture conditions presents a number of experimental challenges, even though lens lineage formation and lens fiber cell differentiation are among the best characterized model systems at both genetic and molecular levels [16, 18, 20, 23, 35, 59, 69, 82]. It has been shown that cultured *lens epithelial cells* can be differentiated into primitive lens-like structures termed "lentoid bodies." Lentoid bodies are 3-D structures that resemble the lens as they are both transparent and refract light. They can be generated in vitro either from primary, spontaneously transformed or viral oncogene-transformed lens epithelial cells [8, 45, 46, 51, 75, 76, 87, 112, 119]. Lentoid body formation can also be found in vivo in vertebrate embryos as a result of spontaneous or genetically engineered mutations in genes that operate in the pathways that control lens formation [56, 61, 101]. Finally, it is possible to transdifferentiate lentoids from retinal pigmented epithelium (RPE) cells [68, 72]. The formation of lentoid bodies in different experimental settings shows that the basic program to establish the 3-D structure of the lens is functional independently on the local environment such as in the absence of optic cup/retinal tissue [106]. Thus, lens differentiation from ES cells in vitro appears to be a feasible goal. This chapter first describes the significance of using ES and iPS cells for better

understanding of embryonic lens development and formation of congenital cataracts. A discussion of how iPS cells can help studies of age-related cataract is also included in "New Model Systems Based on ES and iPS Cell Differentiation to Understand Lens Development and Disease," section of this chapter. "Differentiation of ES Cells into Lens" summarizes the current data on lentoid body formation from human and primate ES cells, and the molecular basis of directed differentiation of human ES cells into lens progenitor cells and lentoid bodies. Finally, "Conclusions and Future Directions" provides a summary of current gaps in lens research and future directions to address these problems.

New Model Systems Based on ES and iPS Cell Differentiation to Understand Lens Development and Disease

Use of ES and iPS cells differentiated into lens cells offers a wide range of experimental approaches to better understand embryonic lens formation and lens fiber cell differentiation. Similarly, cataract-specific iPS cells offer a new array of approaches to evaluate various aspects of human lens homeostasis and identification of novel relationships between cellular processes and their impact on lens transparency.

Modeling of Embryonic Development

Although embryological studies on lens morphogenesis date to the beginning of the twentieth century, and have resulted in a comprehensive understanding of the origin of lens cell lineage, formation of the lens placode, formation of the lens vesicle, cell cycle exit regulation in the posterior compartment of the lens vesicle, lens fiber cell terminal differentiation, lens regeneration in specific amphibians, transdifferentiation of lens from other ocular and non-ocular tissues, and lens evolution in animal kingdom ([18–20, 30, 35–38, 48, 59, 63, 69, 82]), a number of important questions remain to be addressed, with three examples described below.

Based on studies in chicks and zebrafish, it has been proposed that lens progenitor cells originate from a common pool of pre-placodal cells [1, 38, 105]. Data to support this attractive model on mammalian lens development are still missing. A large body of data exists to support the role of FGF signaling at multiple stages of lens development [88]; however, little is known how the specificity of this signaling is established in the embryo in a 3-D space crowded with many signaling molecules, their agonists and antagonists [102]. The lens is also a unique tissue in terms of its terminal differentiation. To achieve transparency, lens fiber cells lose their subcellular organelles including the nuclei in a highly controlled process that ultimately preserves the lens fiber cells for the rest of the life [3, 4]. These questions

can be addressed through the use of ES cell differentiation as described in "Differentiation of ES Cells into Lens" and future experiments outlined in "Conclusions and Future Directions" of this chapter.

Congenital Cataracts

Congenital cataracts are typically caused by mutations in genes that control lens development and by mutations in genes encoding key lens structural proteins [36, 40, 98]. Although molecular mechanisms for many of these genes were established using mouse models, the power to produce lens cells from human patients that carry these mutations is unique. The advantage of this system is that one can prepare human lens cell extracts from genetically defined material and study protein–protein interactions of mutant crystallins and lens membrane proteins in their native environment [17]. Similarly, it is possible to derive lens cells from patients with mutations in DNA-binding transcription factors such as *FOXE3* [69], *HSF4* [11, 26, 100], *MAF* [17, 123], *PAX6* [43], and *PITX3* [7, 10, 96] to study molecular mechanisms of these mutations in their native biological environment. This approach should identify those specific genes with disrupted expression due to specific missense mutations and/or by their haploinsufficiency [17].

Age-Related Cataract

Age-related cataract is a disease of the ocular lens that is responsible for just under half of blindness worldwide, and is expected to increase as a result of extended life spans in industrialized, emerging-market, and underdeveloped countries [71]. Age-onset cataract develops between the age of 40–50 years as a result of the progressive breakdown of the lens microarchitecture [97]. Age-onset cataract is a complex disease involving both genetic and environmental factors that affect 42 % of the population between the ages of 52–64, and 91 % of the population for ages 75–85 [54, 103]. Genetic studies of age-related cataract point to both multiple genes and environmental factors influencing the phenotype [71, 97]. The Beaver Dam Eye Study suggests that mutations in a single gene/locus could be responsible for as much as 35 % of nuclear and up to 75 % of cortical cataract incidence [39, 47, 55]. Other studies using siblings and twins also demonstrate significant genetic influence on age-onset cataract [41, 97].

Age-related (or senile) cataract is defined as cataract occurring in people over the age of 50 in the absence of known mechanical, chemical, or radiation trauma. At the molecular level of age-related cataract, lens structural proteins, the crystallins, become oxidized and water-insoluble, and form high molecular weight aggregates. The continual accumulation of crystallin aggregates and other lens proteins causes opacification and loss of lens transparency. The current treatment

of senile cataract is surgery that replaces the opaque lens with an artificial intraocular lens. Although the surgery is routinely performed in the USA, numbering 1.5–2 million patients treated annually, it represents a major Medicare reimbursement category. It has been estimated that a 10-year delay in the onset of senile cataract could decrease the number of surgeries needed by almost one half, thus significantly decreasing vision care costs ([58]; www.nei.nih.gov/strategicplanning/np_lens.asp). Progress in human cataract research is hampered by the lack of genetically defined and abundant experimental materials as well as the absence of relevant animal models [6, 41]. The use of cataract-specific iPS cells offers a unique opportunity to develop well-defined human cell culture models to study cataract as a disease of lens protein homeostasis.

Differentiation of ES Cells into Lens

In this section, we will first summarize our knowledge about mammalian lens formation that is relevant to the design of experiments to differentiate lens cells from ES cells ("Mammalian Lens Development and Lessons for a Rational Design of ES Cell-Based Differentiation Systems"). We then provide examples of lentoid body formation in various ES culture systems ("Formation of Lentoid Bodies") and describe a procedure to produce highly enriched lens progenitor cells and "immature" lentoid bodies from human ES cells ("Lens Differentiation from Human ES Cells in Chemically-Defined Conditions"). Finally, we will discuss different strategies to improve the differentiation of human lentoid bodies ("3-D Cultures of Lentoid Bodies to Improve Their Differentiation Status").

Mammalian Lens Development and Lessons for a Rational Design of ES Cell-Based Differentiation Systems

Multiple signal transduction systems including BMP (bone receptor protein), FGF, Notch, TGF-β, and Wnt have been identified to control various stages of lens morphogenesis [18, 38, 59, 64, 102]. In addition, the origin of lens lineage from the pre-placodal region shown in chicken and zebrafish models suggests that early stages of the differentiation process require the formation of neuroectoderm and its subsequent "by-product," the pre-placodal ectoderm [105].

Neuroectoderm formation in cell cultures can be induced by a variety of growth factors, inhibitors of BMP signaling including noggin, follistatin, cerberus, chordin, ventropin, and gremlin [2, 92] as well as small drugs such as SB431542 [15]. It has been found recently that noggin is produced by a subpopulation of MyoD-positive cells in the epiblast; their immunologically mediated ablation interfered with lens and optic cup morphogenesis [31].

Loss-of-function studies of *BMP4* in mouse established a critical role of this growth factor for lens placode formation [27]. *BMP7* knockout mice also develop ocular abnormalities that were linked to the abnormal lens induction [65, 116]. In addition, studies of lens formation through conditional knockouts of two BMP receptor genes, *Acvr1* and *Bmpr1a*, further confirmed the essential roles of BMP signaling in lens induction, as reduced lens placode thickening and failure of lens invagination were observed [86]. In ex vivo explant assays using chicken embryonic tissues, BMPs have been shown to specify the formation of lens and olfactory placodes [80, 99]. BMP signaling not only plays a role in the formation of lens placode but also participates in lens fiber cell differentiation. BMP2, BMP4, and BMP7 have been shown to induce the expression of markers of fiber differentiation in primary chick lens cell cultures. In addition, expression of noggin, an inhibitor of BMP signaling, in the lenses of transgenic mice resulted in a postnatal block of epithelial-to-secondary fiber differentiation [9].

Numerous studies have shown multiple functions of the FGF (fibroblast growth factor) signaling pathway for the formation of the lens placode [105]. FGF signaling is well known as the key trigger for lens fiber cell differentiation [63]. The pioneering work conducted more than two decades ago showed that FGF2/bFGF is a potent inducer of lens fiber cell differentiation in vitro [14]. A recent study using conditional triple knockout mice with deletion of FGF receptors, *Fgfr1, Fgfr2*, and *Fgfr3,* provided evidence for the essential role of FGF signaling in lens fiber cell differentiation in vivo. The specific inactivation of these three FGF receptors at lens pit stage totally abrogated lens fiber cell differentiation, resulting in a hollow lens [126]. Transgenic mice expressing a dominant-negative *Fgfr1* in the presumptive lens ectoderm showed many early stage defects including reduced lens placode thickness and delayed lens placode invagination [25]. Studies on two genes, *Frs2* and *Ndst1*, also revealed that FGF signaling is critical for lens placode formation. *Frs2α* encodes a docking protein for linking FGFRs with a variety of intracellular signaling pathways. A mutation of this gene $Frs2\alpha^{2F/2F}$ led to the halt of the lens development at lens placode stage in severely affected mutant eyes [32]. *Ndst1* (*N*-acetylglucosamine *N*-deacetylase-*N*-sulfotransferase 1 enzyme) encodes an enzyme for biosynthesis of heparan sulfate proteoglycans, which is low affinity co-receptor of FGFRs. Inactivation of *Ndst1* in mouse resulted in invagination defects of the early lens [79]. The most recent study showed that inactivation of *Fgfr1* and *Fgfr2* at lens placode stage led to increased cell death and the formation of a thinner lens placode, suggesting that the primary role of autocrine or paracrine FGF signaling is to provide essential survival signals to lens placode cells [29].

Recent genetic experiments, lens-specific inactivation of *Jag1* [60], *Notch2* [93] and *RBP-J* [90] have established role of Notch signaling in primary lens fiber cell differentiation.

Both canonical Wnt signaling, via β-catenin, and planar cell polarity (PCP/Wnt) non-canonical Wnt signaling play a range of roles in lens morphogenesis [64, 67]. Wnt/PCP signaling is required for organization of lens fiber cell cytoskeleton and lens 3-D architecture.

In summary, studies of lens development suggest that active BMP and FGF signaling are required for lens cell formation. FGF signaling is sufficient to induce lens fiber cell differentiation in in vitro cultures, and modulation of this process via Notch, Wnt/β-catenin and Wnt/PCP signaling pathways could provide additional tools to recapitulate lens ontogenesis from ES cell cultures.

Formation of Lentoid Bodies

Three earlier procedures identified lentoid body formation in primate and murine ES cells cultures. These methods were limited to a production of a small percentage of lentoid bodies along with a number of other cells types such as retinal pigmented epithelium (RPE) [44, 77, 107]. The protocols used in these earlier studies employed mouse feeder cells, and differentiation was induced by co-culture with mouse PA6 stromal cells ("SDIA, or cultures"). External FGF2 was added to some cultures [77]. The yield of lentoid bodies was between 200 and 300 colonies/10-cm dish after 30 days in culture. Formation of lentoid bodies was also detected when both mouse and human ES cells were cultured on matrix components of the human amniotic membrane ("AMED system") together with many other cell types including dopaminergic neurons, motor neurons, and RPE cells [111]. These experiments provided the "proof-of-principle" of lens cell formation from mammalian ES cells; nevertheless, they are not suitable for the standardized production of enriched lens cells and lentoid bodies.

Lens Differentiation from Human ES Cells in Chemically Defined Conditions

Using the information on normal lens formation ("Mammalian Lens Development and Lessons for a Rational Design of ES Cell-Based Differentiation Systems"), we established a new experimental three-stage protocol with defined growth factors to generate large quantities of lens progenitor cells and lentoid bodies from human ES cells as shown in Fig. 4.1. Inhibition of BMP signaling by recombinant noggin triggered differentiation of ES cells towards neuroectoderm. Subsequent reactivation of BMP and activation of FGF signaling elicited robust formation of lens progenitor cells marked by the expression of *PAX6* and αA- and αB-crystallins (*CRYAA* and *CRYAB*). The formation of lentoid bodies required the presence of FGF2 and the total number of the lentoids increased in the presence of Wnt3a yielding approximately 1,000 lentoid bodies per a 30 mm well. Lentoid bodies expressed and accumulated lens-specific markers including αA-, αB-, β-, and γ-crystallins, filensin/BFSP1, BFSP2/CP49, and MIP/aquaporin 0 [122]. Nevertheless, morphological and scanning and transmission electron microscopic analysis of these lentoid bodies identified nucleated lens cells and only moderately elongated

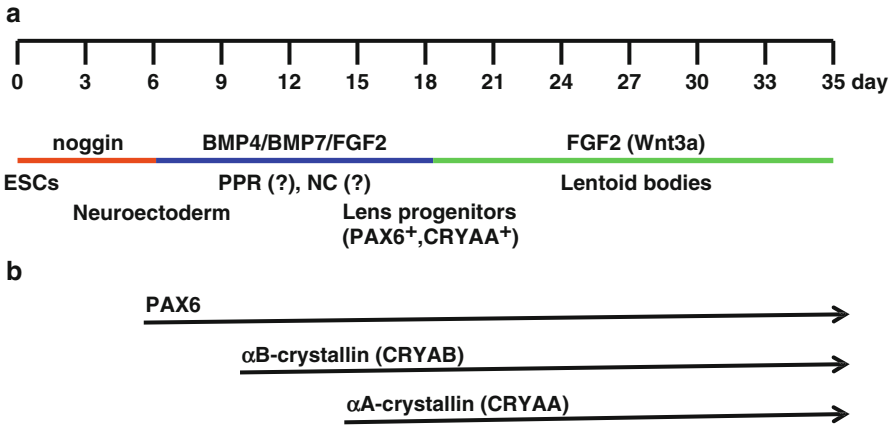

Fig. 4.1 Diagrammatic summary of a three-step procedure to differentiate human ES cells into lens progenitor-like cells and lentoid bodies. (**a**) Diagram of three steps: noggin treatment (days 0–6), BMP4/BMP7/FGF2 treatment (days 7–18), and differentiation in the presence of FGF2 (essential factor) and Wnt3a (modulatory factor) (days 22–35). Formation of putative cell populations including the neuroectoderm, pre-placodal region (PPR) and neural crest (NC) cells is indicated. (**b**) Sequential activation of PAX6, αB-crystallin (CRYAB) and αA-crystallin (CRYAA) indicates establishment of the lens progenitor-like cells around day 14 of the culture. At this time, the number of $PAX6^+$ and $CRYAA^+$ cells was 65 and 41 %, respectively [122]. Both αA- and αB-crystallins accumulate during the differentiation of lentoid bodies

lens fiber cells. These data indicated that while specific pathways of the lens fiber cell differentiation program such as synthesis and accumulation of both αA- and αB-crystallins were turned on in the "immature" lentoid bodies; however, activation of the denucleation pathway was not achieved. We conclude that this procedure can be immediately used to probe various aspects of human lens lineage cell formation focusing on the function of specific DNA-binding transcription factors, chromatin remodellers, and extracellular signaling; nevertheless, follow-up studies are necessary to address the culture conditions to achieve formation of "mature" lentoid bodies comprised of elongated enucleated lens fiber cells.

3-D Cultures of Lentoid Bodies to Improve Their Differentiation Status

A number of potential improvements of the differentiation procedure described above should be considered and empirically tested. In principle, the system can be improved through testing of different 3-D gels and extracellular matrix proteins that are found in the lens capsule, growth of lentoid bodies on lens capsule, specific activators and inhibitors of differentiation, chemical libraries, 3-D scaffolds to generate a gradient of growth factor(s), and any combination of these procedures. In addition, genetically engineered human and mouse ES cells that carry fluorescent

reporter genes, under the control of lens regulatory elements, can be used to aid in the analysis of the differentiation process.

There are at least three commercially available 3-D systems: ExtraCel hydrogel (Glycosan Biosystems), HyStem-C Cell Culture Scaffold kit (Sigma), and Cultrex 3-D Culture Matrix Extract (R&D Systems). Each system allows for the incorporation of variable amounts/ratios of laminin, collagen IV, entactin/nidogen, perlecan, fibronectin, collagen XVIII and sparc/osteonectin, extracellular matrix (ECM) proteins found in the lens [21, 117].

A number of drugs have been shown to promote cellular differentiation with some of the tested in lens cell cultures. These include specific inhibitors of DNA methylation such as 5-azacytidine and 5-deazacytidine [12, 49, 94], inhibitors of histone methyltransferases (cytarabine and decitabine [84]), inhibitors of histone deacetylases (valproic acid and sodium butyrate [22, 24, 34, 74, 78]), and inhibitors of cyclin-dependent kinases (olomoucine and roscovitine [70, 73, 89, 115]). Of particular interest are the rho-kinase (ROCK) inhibitors, Y27632 and PP-1, as the PP-1 drug has been successfully used to promote cell cycle withdrawal and commitment of lens cells to differentiate [113, 114].

Considering the specific roles of Notch and Wnt signaling pathways for lens fiber cell differentiation, and the role of Wnt signaling in the differentiation of lens epithelial cells described above ("Mammalian Lens Development and Lessons for a Rational Design of ES Cell-Based Differentiation Systems"), stimulation of ES cell differentiation may be considered. Recombinant Notch ligands, Jagged 1 and 2, can be added transiently during the thirds stage of the differentiation procedure. Concerning Wnt signaling, the situation is more complex as multiple Wnts and their receptors, the frizzled proteins, can regulate lens development both in the epithelial and fiber cell compartments. Nevertheless, inclusion of Wnt3a improved the quantitative parameters of the current procedure of lentoid body formation [122].

Ongoing experiments in the laboratory are aimed to improve differentiation of lentoid bodies using a combinatorial approach as outlined above. The procedure can be improved via genetically engineered ES cells [5] that carry fluorescent markers under the control of lens regulatory regions from genes known to control different stages of the lens lineage formation, cell cycle exit, and terminal differentiation. For this purpose, the EGFP, or enhanced green fluorescent protein marker can be inserted into a specific BAC clone with PAX6 (early marker), HSF4 (late marker), β-/γ-crystallins, DNase IIβ, MIP/aquaporin 0, paralemmin, and other genes expressed in terminally differentiated lens fiber cells as established for similar differentiation systems [83, 110].

iPS Cells and Cataract Research

For the first time in human lens research, we are about to establish a general strategy to model human lens development and diseases with an *unlimited* supply of lens cells that originate from *genetically and phenotypically defined* human source(s).

Table 4.1 A representative list of distinct nuclear reprogramming procedures to generate human iPS cells

Starting cell type	Treatment	Abbreviation	References
Skin fibroblasts	[*Oct3/4,Sox2,Klf4,Myc*]-retroviruses	iPS	[108]
IMR90 cells, newborn foreskin fibroblasts	[*Oct4,Sox2,Nanog,Lin28*]-lentiviruses	iPS	[125]
Fetal, neonatal, and adult fibroblasts	[*Oct4,Sox2,Klf4,Myc*]-retroviruses + *hTERT* + *SV40LT*	iPS	[81]
Fibroblasts, liver cells	[*Oct4,Sox2,Myc,Klf4*]-adenoviruses	Adeno-iPS	[104]
Terminally differentiated amniotic fluid cells	[*Oct4,Sox2,Klf4,Myc*]-retroviruses	AF-iPS	[28]
Amnion-derived cells	[*Oct4,Sox2,Nanog*]-lentiviruses	hADC-iPS	[127]
Neural stem cells	[*Oct4*]-inducible lentivirus	NiPS	[53]
Peripheral blood mononuclear cells (PB-MNCs)	[*Oct4,Sox2,Klf4,Myc*]-retroviruses + *Htert* + *SV40LT*	BM-iPS	[57]
Umbilical cord matrix and amniotic membrane	[*Oct4,Sox2,Klf4,Myc*]-retroviruses, vitamin C, valproic acid		[13]
Human newborn fibroblasts (HNFs)	Proteins		[52]
Human foreskin fibroblasts	Episomal vector		[124]
Human embryonic fibroblasts (HEF)	*piggybac* transposon		[118]
Human peripheral circulating T cells	Sendai virus	TiPS	[95]

In addition, these materials can be shared between multiple laboratories to accelerate research. The pioneering work of S. Yamanaka at the Institute for Frontier Medical Sciences, Kyoto University, Japan, to establish the reprogramming procedure using skin fibroblasts provided proof-of-principle that the iPS cell can be established from somatic terminally differentiated cells, and these iPS cells behaved like authentic ES cells in a series of functional tests [108, 109]. A large follow-up effort in a number of laboratories worldwide resulted in expansion of the reprogramming procedures and cell types suitable for these manipulations. The majority of currently existing procedures are summarized in Table 4.1. It has been shown recently that iPS cells can be produced from a cataract patient using lens epithelial cells as the starting material [85]. Most importantly, these iPS cells were differentiated into lentoid bodies using the procedure described here (see Fig. 4.1) [85]. Nevertheless, whether iPS cells, generated through other reprogramming protocols and cell types, are capable of producing lentoid bodies similar to those generated from human ES cells, remains to be formally proven.

Conclusions and Future Directions

One of the most pressing objectives of medical research today is to develop novel approaches to model formation of human organs, tissues and diseases. Use of human ES and iPS cells differentiated into individual tissues provides the highest possible promise to achieve this objective as it is now possible to understand the contribution of genetic and environmental factors in various diseases including those related to aging such as age-onset cataract.

Thus, the present cell culture system can be used to modulate these common signaling pathways during lens formation [62] via siRNA technology and through the use of small drug molecules, inhibitors of FGF and BMP signaling (e.g., SB431542—an inhibitor of the Alk1 receptor, SU5402—an inhibitor of FGFR and U0126—an inhibitor of MEK) to study formation of lens lineage and formation of alternate cell fates that originate from the common pre-placodal region [105].

It is now possible to produce iPS cells from human patients that carry heterozygous mutations in regulatory genes such as *PAX6, FOXE3, MAF, HSF4, PITX3*, and others and to identify those genes that are not properly regulated during early stages of lens development. In contrast, studies of cataractogenesis using the system of ES/iPS cells seems be premature until procedures to generate enucleated lentoid bodies with distinct epithelium/fiber cell compartments are established. The long-term benefits of the research to model human cataract using iPS cells should stimulate our efforts to achieve this challenging goal.

Acknowledgements We thank Dr. Louise Wolf for critical suggestions. We are grateful to Dr. Eric Bouhassira and ES cell core facilities at the Ruth L. and Davis S. Gottesman Institute for Stem Cell Research and Regenerative Medicine of the Albert Einstein College of Medicine for their continuous support. Grant support to AC: R01 EY102200, EY014237 and R21 EY020621. The Department of Ophthalmology and Visual Sciences is supported by an unrestricted grant from Research to Prevent Blindness, Inc.

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Chapter 5
Stem Cells and Glaucoma

Jonathan Hertz and Jeffrey L. Goldberg

Abstract Stem cell-based therapies provide new hope for treating glaucoma and other optic neuropathies. Transplanting stem cells or stem cell-derived cells into the retina could provide neuroprotective support to surviving neurons or potentially replace neurons that have already been lost in order to restore visual function. However, before these therapies reach patients, there is a need to identify the appropriate donor cell type(s) to use, as well as how best to differentiate and deliver these cells, to maximize integration, neuroprotection, and functional recovery in the injured retina. Here we review progress towards these goals and critical next steps to bringing stem cell therapies to glaucoma.

Introduction

Glaucoma, the most common neurodegenerative disease of the inner retina and optic nerve, affects more than 60 million people worldwide [1], and results in the dysfunction and death of retinal ganglion cells. The onset of glaucoma is elusive and the progressive degeneration is slow. Because of this, diagnosis often ensues only after

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S.H. Tsang (ed.), *Stem Cell Biology and Regenerative Medicine in Ophthalmology*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-5493-9_5,

cell degeneration and some loss of visual function [1]. Retinal ganglion cell (RGC) loss is irreversible and advancing vision damage leads to bilateral blindness in as many as 14 % of all diagnosed patients [2]. The only current treatment for slowing the degeneration of RGCs has been lowering intraocular pressure (IOP) [3]; however, in some patients, ocular hypotensive-based therapies fail to stop the loss of RGCs and progressive visual dysfunction. Stem cell-based therapies provide new hope for treating glaucoma and other optic neuropathies. Transplanting stem cells or stem cell-derived cells into the retina could provide neuroprotective support to surviving neurons or potentially replace neurons that have already been lost in order to restore visual function. However, before these therapies reach patients, there is a need to identify the appropriate donor cell type(s) to use, as well as how best to differentiate and deliver these cells to maximize integration, neuroprotection, and functional recovery in the injured retina. Here we review progress towards these goals and critical next steps to bringing stem cell therapies to glaucoma.

Stem and Progenitor Cells

Stem cells are defined by two key properties: the ability to self-renew and the capacity to differentiate into multiple cell types [4, 5]. During development, pluripotent embryonic stem cells (ESCs) mature into three distinct germ layers and restrict their cell fate competence to specific progenitor cell lineages [6–8]. ESCs derived from the blastocyst inner cell mass, once in culture, possess nearly unlimited proliferative and self-renewal capacity [4]. One step more restricted, neural stem cells (NSCs) can differentiate into the diverse array of neural and glia subtypes found in the central nervous system (CNS). In the retina, retinal progenitor cells (RPCs) self-renew and remain multipotent but cell fate is limited to retinal neurons and glia [9–12]. Thus, it remains unknown to what extent the degree of lineage restriction or differentiation towards the RGC fate prior to transplantation will maximize either neuroprotection of and/or integration into the glaucomatous retina. By better understanding how RGCs develop, we may recapitulate such signals ex vivo to generate appropriate cell types for either neuroprotective or cell replacement-based therapies in the retina to treat glaucoma.

Retinal Ganglion Cell Fate Determination

RPCs generate all six major types of neurons—rods, cones, horizontal cells, bipolar cells, amacrine cells, and RGCs—and Müller glia found in the retina (Fig. 5.1). Each cell type is functionally and morphologically distinct and resides at a stereotyped location in the retina. Rod and cone photoreceptors absorb photons and convert these signals to electrical signals that are further processed by interneurons

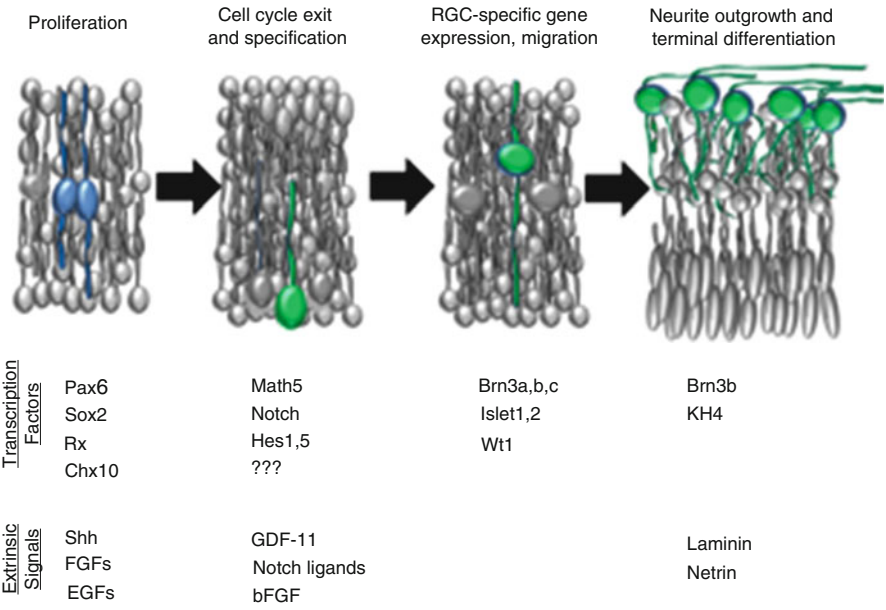

Fig. 5.1 Summary graphic describing the stages of progenitor cell (*blue*) to retinal ganglion cell (*green*) transition, and the transcription factors and extrinsic, environmental signals known to be associated with each stage. *Math5* and *Notch* are known to regulate cell cycle exit but are not sufficient on their own to specify RGC fate (see text and Fig. 5.2)

(amacrine, bipolar, and horizontal cells), which connect synaptically to RGCs. RGCs then carry all visual information to the brain along their axons [13].

How does a developing RGC integrate into its environment? RGCs are among the first neurons to arise from a common pool of multipotent retinal progenitors in the retinal neuroblast during embryonic development. After exiting the cell cycle, RGCs migrate across the retina to the ganglion cell layer (GCL). In the GCL, RGCs extend axons towards the optic nerve head and form the optic nerve which ultimately connects the eye to the brain. RGCs proceed to form synaptic connections with presynaptic amacrine and bipolar interneurons [13–15]. It remains unclear what signals regulate neural integration, or to what degree each step—cell fate specification, survival, migration, neurite growth, and synaptic integration—depends on the preceding one (see Fig. 5.1).

What signals regulate retinal progenitor competence to specify RGC fate determination? Through seminal "birth-dating" experiments, two well-defined but overlapping RPC competence states for generating specific types of neurons, including RGCs, were identified [16]. "Early" embryonic retinal progenitors differentiate into early-born retinal neurons beginning with RGCs and amacrine, cone photoreceptor and horizontal cells [17, 18]. This is followed by an overlapping shift in cell competence to commit to late-born retinal cells, including rod photoreceptors, bipolar, and Müller glia [10]. How these competence changes

occur is not well understood but evidence suggests that these differential competence states are strongly influenced by intrinsic mechanisms. Time-lapse experiments suggest that RPC competence is intrinsically programmed and linked directly to temporal context, albeit with some stochastic component [19]. Heterochronic transplantation experiments in both chick and rodents, in which progenitors from different stages of development transplanted to an environment of a different age/context (either earlier or later) support this premise [20]. For example, early chick progenitors, which normally generate RGCs in vivo, retain their competence for RGCs irrespective of the age of the surrounding environment. Experiments with cultured rat progenitors showed that early progenitors that typically generate RGCs, amacrine cells, and cone photoreceptors do not lose this competence when cultured in different environments known to secrete inductive signals for other neural fates [20]. This suggests that RPC competence to produce distinct types of cells differs depending on the stages of development, independent of environmental context. Similarly controlled spatiotemporal waves of changing cell competence have been observed in many areas of the CNS, including cortex and hippocampus. While early RPCs retain their early-born neuron competence, late RPCs can be influenced by environmental signals. Late progenitors cultured in the presence of early retinal conditioned media were coaxed into the RGC fate, demonstrating that cell competence changes can change in specific directions [21]. Taken together, cell competence for the RGC fate is intrinsic to the early retinal progenitor and decreases during development in a discrete temporal window in order to establish the appropriate cell numbers.

Besides cell competence, cell cycle and cell division mechanisms in retinal progenitors also change over time during development and greatly influence cell number and fate. Cell-cycle duration doubles throughout retinal neurogenesis [22]. Furthermore, the type of cell division shifts over time in retinal progenitors. During early retinal development there is considerable generation of mitotic progenitors, as large numbers of cells divide symmetrically, each giving rise to two progenitors. As development progresses the generation of new progenitors decreases, concomitant with the increased generation of post-mitotic neurons. Cell polarity and the orientation of the cell division plane correlate with proliferation and cell determination in the developing cortex and in the retina [23]. Thus, asymmetric segregation of cell-fate determinants during cell division may play an important part in generating cell diversity in vertebrate retina. For example, the asymmetric segregation of the protein numb, occurring only in a precise cell division plane, plays a role in cell fate determination in the rat retina [24]. Thus, cell competence for specific neural subtypes, including RGCs, is dynamically regulated by cell autonomous, environmental, and cell polarity signals which work in concert to define the temporal window in which specific cells are born. As reviewed below, disruption of any of these signals has adverse effects on retinal development.

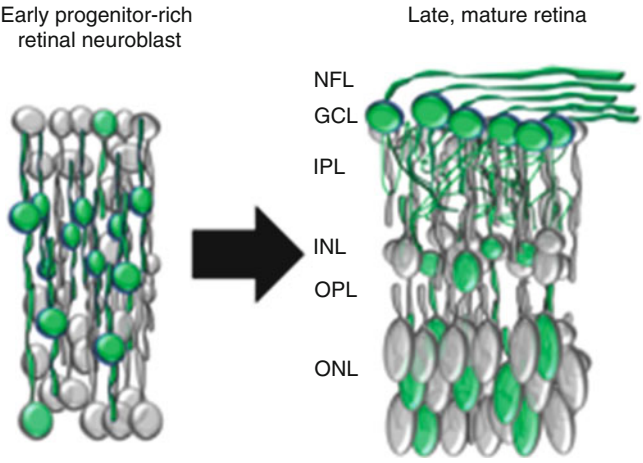

Fig. 5.2 Math5-positive progenitor cells (*green*, *left*) differentiate into RGCs and many other retinal neurons found in the mature retina (*green*, *right*), suggesting that *Math5* is not sufficient on its own to specify RGC fate. (*NFL* nerve fiber layer, *GCL* ganglion cell layer, *IPL* inner plexiform layer, *INL* inner nuclear layer, *OPL* outer plexiform layer, *ONL* outer nuclear layer)

Transcription Factors Regulate RGC Fate

Through numerous gain and loss of function experiments both in vitro and in vivo, transcription factors and secreted factors have been shown to regulate the specification and differentiation of RGCs, but precise instructive signals remain unknown (Figs. 5.1 and 5.2). On the cell-autonomous side, transcription factors are master regulator proteins which regulate expression of downstream gene targets. Transcription factors, particularly the basic helix loop helix (bHLH) and homeodomain families, have been shown to control the differentiation and patterning of many of the diverse cell types in the CNS, and specific gene regulatory pathways are required to complete and progress through a series of developmental stages. In a hierarchical manner, transcription factors regulating early developmental processes are important early, while transcription factors regulating terminal differentiation and later maturation processes are important later. Some of the main transcriptional regulators within this hierarchy, including *Pax6, Six3, Rx, Chx10, Notch, Ath5*, and the *Brn3* family of transcription factors, have been identified and examined extensively, but the genes these transcription factors target and how these signals work in together remain unknown.

Pax6

Loss-of-function studies with knockout mice have placed certain homeobox-containing transcription factors at the top of gene regulatory networks controlling

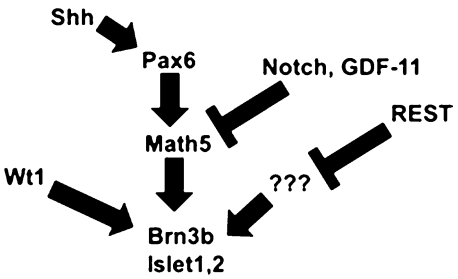

Fig. 5.3 General overview of known factors regulating retinal ganglion cell fate specification. See text for details

retinal development. These genes include *Pax6*, *Rx*, *Chx10*, and *Six3*, which are all expressed in RPCs in the retinal neuroblast. These homeobox genes are expressed in all RPCs in the beginning of retinogenesis and are required for the specification of RGCs as well as other retinal cell types. *Pax6* is required for eye field specification during the early stages of eye development [25] and also for generating cell types in the developing retina [26]. In seminal gain of function experiments in drosophila, later shown in vertebrates, *Pax6* was sufficient to trigger the cascade of signals required for eye formation [25]. Conversely, elimination of *Pax6* function by a conditional knockout in the developing retina led to a loss in the specification of all retinal cell types excluding amacrine cells. *Pax6* functions, at least in part, to promote the expression of the bHLH proneural genes in retinal neuroblasts, as loss of *Pax6* results in the decreased expression of genes encoding the proneural bHLH factors *Ath5*, *Ath3*, and neurogenin [26]. NeuroD expression is unaffected by the loss of *Pax6*, consistent with evidence from knockout mice establishing that NeuroD is crucial for amacrine cell differentiation [27]. Taken together, these data demonstrate that multiple sets of transcription factors regulate RPC proliferation and differentiation during retinal neurogenesis, which begins with RGCs, and corresponds with the upregulation of Ath5 expression.

Ath5

Through loss-of-function experiments in mouse, the proneural bHLH gene *Ath5* (also called *Xath5* in Xenopus, *Cath5* in chick and *Math5* in mouse) was demonstrated to be necessary but not sufficient for RGC fate (Fig. 5.3). During development, *Ath5* is expressed almost exclusively in the retina. In the mouse retina, *Math5* expression begins directly before the birth of the first RGC and its expression decreases in daughter RGCs soon after RGC precursors exit the cell cycle [28]. The importance of *Ath5* in the RGC lineage was recognized through both gain- and loss-of-function studies. Overexpression of *Cath5* in chick retinas [29] and *Xath5* [28, 30] in Xenopus retinas stimulates RGC production at the cost of generation of other retinal cell types. Null mutations in *Ath5* in mice [31, 32] and zebrafish [33] lead to almost complete absence of RGCs. Although *Ath5* is essential for RGC formation, evidence suggests that it is probably upstream of the instructive signals for RGC specification, as lineage-tracing experiments show that

Ath5-expressing RPCs give rise to multiple cell types [31]. *Ath5* likely acts as a proneural gene to promote the establishment of a field of progenitor cells that are competent to turn into RGCs but not to specify the RGC lineage. *Ath5* overexpression, which increases the number of RGCs, may do so by generating a larger pool of RGC progenitors competent to then differentiate into RGCs. Taken together, evidence suggests that *Ath5* is necessary but not sufficient to specify RGC fate, and that *Ath5* may not specify a particular cell type at all, but rather is more involved in multiple steps of retinal neurogenesis, including the differentiation of RGCs and other cell types in the retina.

Notch

During development, pro-neurogenic signals compete with opposing signals to coordinate the generation, distribution and patterning of newly born neurons. Opposing RGC fate, *Notch* has been shown to be a key regulator of cell fate in the CNS and plays an important role as a negative regulator of RGC production [34, 35]. The *Notch* signaling pathway is activated by the binding of ligands such as Delta, typically through neighboring cell–cell interaction. Activation of this pathway leads to the proteolytic cleavage of *Notch* and the release of a *Notch* intracellular domain (NICD). NICD translocates to the nucleus and binds to the highly conserved DNA-binding transcription factor CSL to activate target genes, including the Hairy-Enhancer of Split (HES) family of bHLH genes [36, 37]. Evidence from Drosophila studies demonstrates that the *Notch* pathway negatively regulates neurogenesis in the developing eye through lateral inhibition resulting in the repression of the proneural bHLH gene *atonal* [38]. In the vertebrate retina, the *Notch* pathway is similarly positioned at the top of the regulatory hierarchy in RGC generation and inhibits in RGC production through lateral inhibition. *Pax6* and the *Notch* pathway compete with each other in regulating downstream genes required for the generation of the RGC lineage. *Pax6* is required for the activation of *Ath5* [39], which is required for the RGC lineage. Conversely, *Notch* signaling inhibits *ath5* expression through its downstream target transcription factors, Hes1 and Hes5. It is unknown whether *ath5* is a direct transcriptional target of *Pax6* and/or *Notch-CSL*, or whether other regulating signals are required in these pathways. Taken together, these data suggest that *Pax6*- and *Notch*-dependent mechanisms, in concert with other signals, fine-tune the proper levels of *ath5* expression in a subset of progenitor cells that become competent for RGC specification.

Brn3

Downstream of these pathways, Brn3 proteins (also called XBrn3 in Xenopus) Brn3a, b, and c are class IV POU domain transcription factors and one of the initial and most specific markers for RGC differentiation during development [40]. Around 80 % of RGC precursors express *Brn3b* immediately after cell cycle exit,

and 24 h later the closely related *Brn3a* and *Brn3c* genes are expressed in ~80 and ~20 % of developing RGCs, respectively. These latter two subsets of RGCs significantly overlap the subset of *Brn3b* expressing RGCs [41–44]. These transcription factors have been shown to control dendritic stratification, axonal structure and target selection during the terminal differentiation stage and their expression patterning may control the development of unique subtypes of RGCs (see Fig. 5.1) [45].

Of the Brn3 proteins, *Brn3b* has been best studied, and gain- and loss-of-function experiments indicate that *Brn3b* is directly downstream of *ath5* in the regulatory hierarchy for RGC differentiation. Although *Brn3b* can promote the expression of certain RGC markers when overexpressed [46], there is strong evidence that demonstrates that *Brn3b* itself is not a required cell fate specification gene for RGCs. In *Brn3b*-null retinas, the number of RGCs born initially resembles that observed in wild-type retinas [40]. Therefore, this suggests that there are probably unknown gene regulatory pathways that function in parallel to *Math5* and upstream of *Brn3b*.

In *math5*-null retinas, *Brn3b* expression is greatly reduced [31, 32], consistent with the absence of RGCs. However, it remains unknown whether *Math5* directly regulates *Brn3b* expression. In the retina, the spatial and temporal expression patterns of *Math5* and *Brn3b* are largely non-overlapping. Furthermore, *Math5* is expressed in proliferating progenitor cells and *Brn3b* is expressed in postmitotic RGC precursors and mature RGCs [31, 40]. If *Math5* directly upregulates *Brn3b* expression early on, other mechanisms must be responsible for maintaining high levels of *Brn3b* expression after *Math5* expression declines during retinal development. In *Brn3b*-null retinas, lacZ knocked into the *Brn3b* locus mirrors the normal expression pattern of *Brn3b* [40]. These findings suggest that maintenance of *Brn3b* expression is not likely controlled by autoregulation. It is possible that genes required for RGC specification lie in between and/or parallel to *Ath5* and *Brn3b* in the regulatory hierarchy and that these unknown specification genes either collaborate with or function independently of *Ath5* to regulate expression of *Brn3b*. Interestingly, knocking out both *Math5* and the transcriptional repressor RE-1 silencing transcription factor (*REST*) in mouse retina leads to the generation of ectopic *Brn3b*/*Islet1* double-positive RGCs [47]. This further demonstrates that *Brn3b* expression does not depend entirely on *Math5* expression. Currently it is unknown if REST is repressing uncharacterized cell fate specifying transcription factors, but evidence from experiments in other parts of the CNS suggests this hypothesis [47].

Wt1

The Wilms' tumor gene (*Wt1*), which encodes a zinc-finger transcription factor, was found to function directly upstream of *Brn3b* in the retina [48]. *Wt1*-null mice have a major loss of RGCs in the retina which, similar to *Brn3b*-null mice, initially generates RGCs that are later lost by apoptosis. *Wt1* expression does not overlap

with *Ath5* expression and it is not clear whether *Ath5* regulates *Wt1*. *Wt1*-dependent activation of *Brn3b* could be part of an *Ath5*-independent signaling cascade regulating RGC differentiation. Although *Brn3* family members and *Wt1* transcription factors do not play role in specifying RGC fate from RPCs, it is likely that they signal important downstream targets for the full RGC phenotype, which may need to be upregulated in stem cell-derived RGCs if transplantation is to be considered (discussed further below). Thus there remains a gap in our understanding of RGC fate regulation between upstream transcription factors like *Ath*5 and *Pax6* that are necessary but not sufficient, and downstream transcription factors required for RGC maintenance or survival.

Secreted Molecules Regulate RGC Fate in Concert with Transcription Factors

During development, secreted molecules from the local environment work in concert with transcription factors to induce commitment to subsequent steps in differentiation [49]. Secreted factors such as fibroblast growth factors (FGFs), sonic hedgehog (Shh), and transforming growth factor beta (TGF-β) superfamily molecules have been shown to regulate cell number and the timing of neural differentiation by regulating transcription factor expression [50–52].

Basic Fibroblast Growth Factor

The trophic factor and mitogen basic fibroblast growth factor (bFGF) has been shown to potentiate RGC fate determination in mammalian retinal progenitors [53, 54]. In an RPE transdifferentiation assay, bFGF elicits the expression of RGC marker RA4, although the extent of differentiation may be very limited [29, 55], because those cells did not express many other RGC markers. Expression of these markers was detected in bFGF-primed RPE cultures infected with RCAS–*Ath5* or RCAS–*NSCL1* [29], suggesting that the bHLH hierarchy may integrate input from bFGF to promote RGC differentiation and development. Consistent with previous findings that FGF promotes the retinal neurogenic pathway [53, 56], blocking FGF receptor activation in chick interferes with the progressive wave RGC differentiation from the central retina towards the periphery [57].

Sonic Hedgehog

Sonic hedgehog is another extrinsic factor shown to regulate proliferation and RGC generation and differentiation [58–61]. Recent studies have established a mitogenic role for Shh signaling in CNS progenitor cells. For example, cerebellar granule cell

precursors depend on Shh secreted by Purkinje cells to proliferate in vitro and in vivo [50, 62, 63]. In early retinogenesis, Shh derived from the first-born RGCs promotes propagation of the neurogenic wave front [59] but suppresses RGC genesis as these neurons accumulate, as discovered in zebrafish [59]. Shh secreted by RGCs appears to also negatively affect RGC generation through a different feedback system [64]. Thus Shh regulates the precise number of RGCs generated during development through at least two mechanisms. It remains unclear as to whether the morphogenic property of Shh observed in other areas of CNS development plays a role in regulating these contrasting modes of function.

Shh signals also appear to influence the growth and trajectory of RGC axons [65, 66]. In zebrafish, reduction of Hh activities affects differentiation of late cell types including Müller glia, bipolar cells, GABAergic amacrine cells, and photoreceptors [61, 67]. Furthermore, laminar organization of the retina is disrupted in Shh mutants [67, 68].

Recently, Shh has also been implicated in adult neural stem cell proliferation [69]. Mice with a single functional allele of the Shh receptor patched have an increased percentage of proliferating cells in their retinas throughout the first postnatal week. In addition, the mice have a population of dividing cells at the retinal margin reminiscent of the ciliary marginal zone (CMZ) of lower vertebrates [70]. This suggests that Shh signaling is important for controlling retinal progenitor proliferation and may regulate adult neurogenesis in the mammalian eye. Taken together, Hh signaling, perhaps due to its morphogenic properties, is fundamentally important to many facets of RGC differentiation, including postembryonic ocular growth, but how these mechanisms function together remains unknown.

Growth Differentiation Factor 11

In the retina, feedback regulation of neural cell number, mediated by secreted factors, has been shown to alter the fates of multipotent progenitor by controlling the timing of transcription factor expression. For example, the secreted TGF-β molecule growth differentiation factor 11 (GDF11) negatively regulates the number of neuron generated by controlling the period during which retinal progenitor cells are competent to produce particular progeny. The GDF11 KO mouse has aberrantly persistent *Math5* expression throughout postnatal development resulting in the generation of excessive numbers of RGCs. [52]. Conversely, exposing retinal explants to GDF11 results in the decrease in *Math5* expression resulting in retinas with less RGCs. It is currently unknown which cell type(s) secrete GDF11 as well as whether other GDFs play roles in retinal development. Manipulation of these signaling pathways could provide insight into improving the methods for the generation of donor RGCs for transplantation.

Cell Choices for Transplantation

What types of stem or progenitor cells can be used for RGC therapies in glaucoma or other optic neuropathies? The most comprehensively studied donor cell candidate for cell-based therapies in the retina has been embryonic stem cells (ESCs), which proliferate, self-renew and differentiate into all cell types. In culture, ESCs retain all of these features. ESCs have been differentiated in culture into most retinal cell types including RGCs [71–73].

However, transplantation studies of undifferentiated ESCs have demonstrated that these cells fail to receive the proper instructive cues for correct cell fate specification. Transplanting an uncommitted stem cell will rely heavily on the host tissue to provide differentiation cues to the grafted cells and so far for RGCs, this has yielded only minimal re-integration with no evidence for any functional restoration. Further limitations to using ESCs directly come from observations in some studies in which the cells formed tumors due to uncontrolled proliferation following transplantation [74]. Other challenges may include immune rejection, teratogenic properties, and ethical concerns over the cell source. Thus, control of proliferation and differentiation of these cells is critical before ESCs are safely and ethically used as a cell source for therapeutic transplants.

Neurons and neural stem cells (NSCs) induced from ESCs and transplanted into the injured eye may show more promise for retinal integration [75–78]. Similarly, ESCs differentiated into retinal stem cells (RSCs) as well as various neuronal phenotypes by exposure to pro-neural differentiation factors in vitro prior to transplantation were investigated in a retinal transplantation model [79, 80]. RSCs subretinally transplanted into young mice survived, migrated, integrated, and differentiated into retinal cell types, particularly rod photoreceptors. However, in adults, transplanted RSCs preferentially expressed RGC or glial markers. These findings provide evidence that RSC differentiation following transplantation hinges on the pre-transplantation condition of both the donor RSCs and the host retina.

NSCs from other areas of the CNS, particularly forebrain, have also been investigated as potential donor cell sources to the retina. Upon transplantation to the retina, forebrain-derived NSCs survive, express some retinal cell specific markers, and exhibit retinal cell-like morphologies. Overall, the rate of integration was low and many of the cells were localized in aberrant retinal layers. As with RSCs, the extent of differentiation and integration depend heavily on the age and type of injury to the host retina [81]. Hippocampal-derived NSCs incorporate into injured retina and differentiate into both microtubule-associated protein 2 (map2) positive and GFAP-positive cells, suggesting differentiation into both major types of cell lineages. Although neurons and glial markers were present, no retinal-specific subtype markers were observed, demonstrating that the local retinal environment, either normal or injured, is not sufficient to coax hippocampal NSCs towards retinal cell types.

Bone marrow stem cells (BMSCs), which are far more easily attained than ESCs, also have restricted potential and offer potential therapeutic promise [82–85]. Even

though BMSCs are not linked to neural lineages, they have been coaxed to produce neuron-like cells which express some retinal markers [86]. Following transplantation into the subretinal space, BMSCs generate progeny that express limited retinal markers [87]. However, there is substantially more promise in using BMSCs to produce blood vessels which may be useful to replace vasculature lost in various retinal diseases, or to support the survival of degenerating neurons. For example, following transplantation of BMSCs into the retina, profound revascularization of retina was observed, which resulted in enhanced survival of retinal neurons in models of retinal injury [88–90]. Evidence from these studies further suggests that the improved circulation in these ischemic animal models provide an enhanced conduit for trophic factor delivery which can enhance neuroprotection.

The adult human eye itself contains progenitor cells, which may be influenced towards RGC fate [91, 92]. In lower vertebrates, such as teleosts, the ciliary marginal zone (CMZ) contains stem cells which persist following development and generate new neurons in the continually growing adult teleost retina [93]. Similarly, cells in the ciliary body and a subpopulation of Müller cells in the human retina have been shown to exhibit stem cell-like properties [94–96]. In injury models in lower vertebrates, Müller cells generate new RGCs that then regenerate their axons down the optic nerve [97, 98]. In mammals, neither ciliary body nor Müller cells proliferate in response to retinal injury. However, they display proliferative and multipotent capacity in vitro [99], and in adult mice, optic nerve injury by transection or crush increases cell proliferation and the expression of RPC markers in both the ciliary body and in Müller glial cells and astrocytes in the retina [100, 101]. Thus, stem cells residing in adult tissue, when expanded in vitro and differentiated into the appropriate cells, may enable autologous transplantation-based therapy by using the patient's own eye as the donor cell source.

Another source of stem cells that could be patient-specific is induced pluripotent stem cells (iPSCs). Through reprogramming adult somatic cells back to an embryonic stem cell-like state, iPSCs were first generated and described in 2006 [102–104]. These cells have advantages over ESCs as a potential donor source including obviating ethical debate over ESC use. In similar differentiation paradigms used for ESCs, iPSCs demonstrated the capacity to differentiate into RPCs, and all the retinal cell type progeny including RGCs [105–107]. However, extensive research still needs to be done to eliminate some of the safety issues associated with these cells [108]. For example, because iPSCs can be developed from a patient's own somatic cells, initially it was expected that treatment with iPSCs would circumvent immunogenic responses; however, recent evidence has challenged this notion [109]. Furthermore, the specific methods for reprogramming adult cells to obtain iPSCs may pose significant risks that could limit their clinical use. For example, if viruses are used to reprogram cells in iPSCs, the expression of oncogenes may simultaneously be activated. Recently, generation of iPS cells without introducing genes but rather with repeated treatments of specific proteins was shown to be an effective method for reprogramming somatic cells [110]. Whether iPS cells can differentiate into functional replacement cells for use in

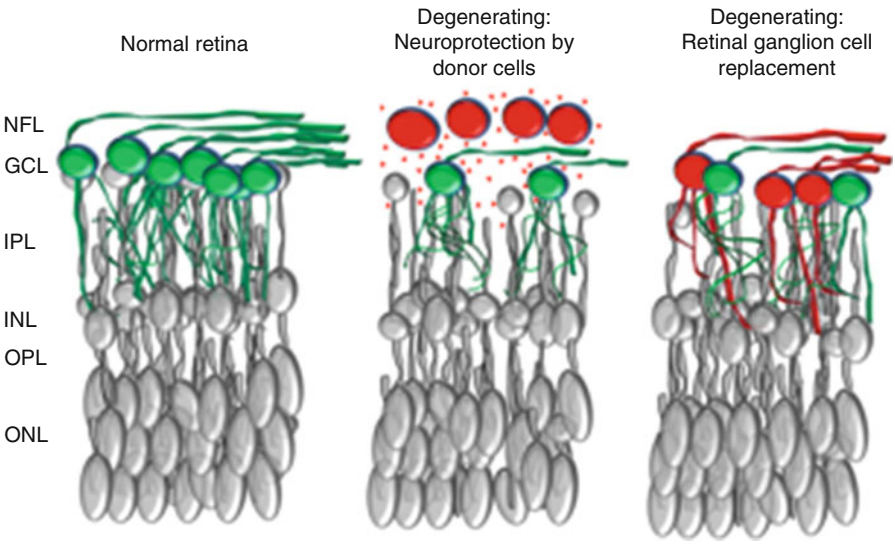

Fig. 5.4 Retinal ganglion cells RGCs (*green cells*) degenerate and die in glaucoma and other optic neuropathies (*middle* and *right*), leaving fewer RGCs than in the normal retina (*left*). Stem cells or cell therapies (*red*) for glaucoma or other retinal ganglion cell degenerations could be used for neuroprotection of residual through trophic support (*red triangles*) or for cell replacement therapy (*right*). (*NFL* nerve fiber layer, *GCL* ganglion cell layer, *IPL* inner plexiform layer, *INL* inner nuclear layer, *OPL* outer plexiform layer, *ONL* outer nuclear layer)

the treatment of glaucoma or other progressive diseases specific to the visual system remains unknown and certainly an important area for investigation.

Thus a number of stem cell sources may be available for therapeutic development for glaucoma. They may have different advantages and disadvantages including accessibility, reproducibility, patient-specificity, and importantly potential for toxicity. As important will be figuring out what capacity each has to help in RGC degenerative disease, and for that, stem cells may have two important uses: replacing RGCs, which will require differentiation and integration into the retina and visual pathway, or protecting RGCs from death, neuroprotection (Fig. 5.4). Next we address progress being made on these two fronts.

Cell Transplantation for RGC Replacement

The majority the research on retinal cell transplantation has concentrated on pathologies involving photoreceptor degeneration [76–78]. Lessons from recent studies on photoreceptor replacement approaches suggest that cells further along in differentiation may be more promising than stem and progenitor cells in neuronal cell replacement therapy [111–114]. For example, MacLaren et al. transplanted dissociated retinal cells, including progenitors and post-mitotic retinal cells, from

various donor ages subretinally in mouse and found that that the post-mitotic rod precursors rather than multipotent progenitors were capable of synaptically reintegrating in the photoreceptor layer. They found that donor cells from ages which marked the peak birthdates of rods had the most profound highly structured morphological and synaptic integration. The transplantation of Nrl+ immature rod cells was capable of improving visually evoked potentials in genetic models of mouse photoreceptor degeneration. Thus, we are only beginning to understand the importance of the spatiotemporal state of a cell which can be exploited to generate donor cells with the greatest potential for neuroprotection and/or integration.

Compared to photoreceptors, there has been less progress made in integrating donor cells for RGC replacement. Transplantation of retinal progenitors from various donor ages do not appear to generate newly born and integrated RGCs in vivo [115–119]. Unlike photoreceptors, RGCs extend lengthy axons to specific targets in the brain in addition to making complex dendritic connections with their synaptic partners in the retina. Additionally, in order to be clinically applicable, enhancing graft integration by altering the host retina must be accomplished without disturbing regular retinal function. To add to the complexity, there are many different types of RGCs, each with highly specialized properties which coordinate complex visual functions and likely draw on synaptic plasticity for wiring during development. Successful replacement of RGCs may require differentiation into specific cell subtypes with highly specialized properties, the establishment of numerous synaptic inputs, and the extension of an extremely long axon to precise brain targets in a manner that preserves the retinotopic map. As such, various groups are currently trying to understand how to coax cells in vitro to produce cells that are further along in differentiation, on the potential premise they may be a more transplantable cell source [114]. Presently, it has not been addressed whether purified RGCs, obtained acutely from the retina or derived from stem cells in vitro, can integrate into the normal or injured adult retina, or at what age or stage during post-mitotic development maximizes donor RGC integration following intraocular transplantation.

First, however, effective delivery of these cells is required before any of these complex set of processes is accomplished. Can cells transplanted into the vitreal surface of the retina get to the ganglion cell layer? Through an abundant array of transplantation studies, intravitreally transplanted cells have been shown to migrate in very close proximity with the inner retinal surface but rarely progress past the inner limiting membrane (ILM) [120]. Peeling away the ILM prior to transplantation results in a dramatic increase in the migration of engrafted cells into the retina [120]. This suggests that a major impediment to cell migration exists within the ILM. Is it the extracellular matrix or the Müller glial endfeet? By degrading various component of the ILM selectively, it was determined that the integrity of the inner basal lamina is neither required nor sufficient to stop grafted-cell infiltration into the retina. In contrast, suppression of Müller cell reactivity dramatically enhanced graft integration [120]. Is migration or neurite growth inhibited in the adult retina, for example by signals found elsewhere in an adult inhibitory CNS environment, such as chondroitin sulfate proteoglycans? For example, treatment with chondroitinase

ABC digests chondroitin sulfate proteoglycans (CSPGs) and promotes neurite outgrowth in the spinal cord and in the retina [121, 122]. Thus, achieving optimal neural integration may require manipulating the host retina either prior to, in conjunction with, or following cell transplantation to create a more permissive environment.

Although there has only been limited success in delivering cells to the inner retina, many developmentally expressed molecular signals persist in the adult retina, including netrin, an RGC axon chemoattractant [123]; N-CAM [124]; and laminin along Müller glial endfeet in the nerve fiber layer [125]. During development, these signals coordinate intra-retinal axon pathfinding as well directing axons to their targets in the brain. These factors may provide the signals sufficient for supporting the growth of new neurite fibers towards the optic nerve head and perhaps even towards targets in the brain. Therefore, the persisting presence of these developmentally critical signals is promising and could potentially be exploited to signal newly integrated immature donor cells as occurs during development.

In order for newly integrated neurons to make communicate and make functional connections with the host retina, synapses must be formed between these cells. Signals for RGC synapse formation such as thrombospondin [126] may be downregulated during normal development but may be reexpressed in an injured retina. This suggests that many of the players involved in the complex wiring of the retina during development may still be exploited to guide the incorporation of new neurons following transplantation. Combinatorial therapies that enhance migration, neurite growth, and synaptogenesis may be required to capitalize on the integration potential of transplanted cells.

Does the degenerating retina enhance integration of donor cells through signaling changes? Targeted apoptotic neurodegeneration has been used to produce highly controlled spatially and temporally specific cell death of selected types of projection neurons within defined regions of the cortex. Photo-activated induction of cell death in the neocortex has been shown to affect migration and differentiation of transplanted neurons as well as transplanted neural precursors [127–131]. In these experiments, later-stage and region-specific immature neurons integrated when transplanted back into injured adult cortex where they usually are located more efficiently than after transplantation to ectopic regions of injured cortex. However, at postnatal stages of development, limits in the survival of the donor, immature cortical neurons offset this improved efficiency [132]. Thus, it remains largely unknown how the glaucotamous retina responds to cell-based therapies compared to normal retina but understanding the changes following injury will provide insight into answering some of these questions.

Cell Transplantation for RGC Neuroprotection

Cell-based neuroprotective therapies geared to providing nourishment and support to surrounding host neurons are more straightforward compared to cell replacement therapies which attempt to replace cells and functionally reintegrate into neural circuits. To provide a neuroprotective benefit, transplanted cells must survive and secrete trophic factors into the host tissue. There is strong evidence that demonstrates that intraocular cell transplantation could benefit a variety of optic neuropathies by providing trophic support to surviving tissue or by encouraging endogenous neuroprotective pathways [77]. Cellular therapy could provide long-lasting and potentially chronic neuroprotection, a potential advantage over pharmacological approaches that require more frequent dosing. Furthermore, specific cues could be exploited to guide stem cell migration to appropriate areas for focal delivery with far better resolution than pharmacological injection. Complex contact-mediated mechanisms, which would be difficult to mimic synthetically, could be exploited to further support and protect persisting neurons in optic neuropathies. Such stem cell behavior has been observed in various neuropathological models, and has been particularly well-characterized following stroke [133, 134].

In addition to supplying trophic factors, transplanted cells may also be able to modify the pathological environment to promote neuronal survival. As an example, stem cells derived from the subventricular zone have been found to modify the local environment directly through immunomodulatory mechanisms [135] or by influencing gene expression in surrounding neurons [136]. In addition, integration of glial precursor cells, which possess active glutamate transporters, into organotypic spinal cord cultures enhanced glutamate uptake and reduced motor neuron cell death, possibly through reducing glutamate excitotoxicity [137]. Furthermore, unlike in the peripheral nervous system, CNS neurite outgrowth following injury is very limited. This lack of regeneration after injury appears to be due to the combination of a lack of neurotrophic signals in the adult CNS, which promote regenerative growth, and inhibitory cues in the CNS environment. The production and release of neurotrophic factors by neural stem cells promotes axonal regrowth in the adult injured spinal cord [138] and, therefore, release of neurotrophic factors by engrafted cells might have beneficial consequences beyond neuroprotection alone. As mentioned earlier, BMSCs, in numerous studies, provided trophic support resulting in increased neuronal sparing in multiple injury models although the mechanism(s) at work remain only speculative. Perhaps combinational cell type therapies consisting of neuronal-induced cells and BMSCs and will work in concert to produce and efficiently deliver trophic factors to degenerating neurons.

Advances in the efficacy and safety of gene delivery to stem cells have increased interest in generating genetically modified stem cells donor cells which can be designed to secrete even more neuroprotective factors. Outside the retina, evidence from a Parkinson's disease mouse model demonstrates that the engraftment of neural stem cells engineered to express GDNF was found to improve the degeneration of dopaminergic neurons following injury [139], which resulted in significant and lasting improvements in the behavioral impairments associated with this injury

model. A number of groups have now demonstrated substantial protection by engineered stem cells in various models of ischemic disease. For example, a significant improvement in neurological degeneration was observed in a rat transient focal cerebral ischemia model following the engraftment of neural stem cells, modified in vitro to express VEGF compared to naive NSCs [140]. Furthermore, the transplantation of human neural stem cells overexpressing VEGF into the cortex overlying an intracerebral hemorrhage lesion has been shown to improve survival of engrafted cells, stimulate host angiogenesis, and recover functional loss in mice [141]. In a similar set of experiments, the introduction of mesenchymal stem cells (MSCs) transfected to express brain-derived neurotrophic factor (BDNF) after permanent middle cerebral artery obstruction was found to reduce lesion size and improve function [142]. Furthermore, in this model, stem cells engineered to produce BDNF provided greater neuroprotection than that observed following the delivery of naive cells. While these techniques are yet to be applied to model of glaucoma, there is new evidence which demonstrates that the transplantation of BDNF-secreting MSCs provides neuroprotection in chronically hypertensive rat eyes. Further investigation will need to be done to see whether this attractive therapeutic approach holds promise the treatment of chronic neurodegenerative retinal and optic neuropathies.

Conclusions

Thus stem cell transplantation provides new therapeutic avenues to combat the irreversible loss of RGCs associated with glaucoma. For treatments to reach patients many obstacles, including the regulation of differentiation, integration, and lasting survival, as well issues regarding efficacy and safety must be overcome. For now, the retina enables an accessible window into important questions about how cell-based therapies could be harnessed to fight neurodegeneration throughout the CNS. With deeper understanding of the cell and molecular basis of these complex processes, the true potential of the stem cell-based therapy in retinal repair will be realized, and with time and careful consideration, transitioned into the clinic.

Acknowledgements This work was supported by the Department of Defense, the Hope for Vision foundation, the National Eye Institute (P30 EY014081, Miami), and an unrestricted grant from Research to Prevent Blindness. JH was supported in part by NIH T32-NS07492, and by a Lois Pope Life Fellowship. JLG is the Walter G. Ross Distinguished Chair in Ophthalmic Research.

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Chapter 6
Bone Marrow Stem Cells in Retinal Disease

Louis K. Chang

Abstract Bone marrow contains populations of self-renewing, pluripotent stem cells, here termed bone marrow stem cells (BMSCs). Mobilized by vascular endothelial growth factor, these play a crucial role in the revascularization of ischemic tissues and organs. Research on mouse models has shown that bone marrow-derived cells contribute to revascularization and improvement in various ischemic tissues, and migrate naturally to damaged sites in the eye. The transplant of BMSCs holds promise for treating ischemic retinal diseases such as diabetic retinopathy. Current treatments to prevent severe vision loss associated with these diseases include panretinal photocoagulation, which decreases the production of angiogenic factors such as VEGF by ablating ischemic peripheral retina, and anti-VEGF intravitreal pharmacotherapy, which temporarily neutralizes VEGF. Unlike these current therapies, revascularization of ischemic retina with therapeutic angiogenesis could prevent sequelae of severe ischemic retinopathy and potentially improve retinal function in a durable, nondestructive manner.

Introduction

Adult bone marrow contains distinct populations of self-renewing, pluripotent stem cells. The most prominent are hematopoietic stem cells (HSCs), which give rise to all lineages of blood cells, including myeloid and lymphoid lineages. HSCs are defined by expression of CD34/DR+/lin− in humans [1] and SCA-1+/Thy-1low/lin− in mice [2, 3]. Endothelial progenitor cells (EPCs) represent a population of pluripotent stem cells capable of differentiating into vascular endothelial cells. The expression of CD34, VEGFR2, and CD133 in humans [4, 5] (and Sca-1+/c-Kit+/lin-/VEGFR2+ in mice) is often used to define EPCs, although definitions vary

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S.H. Tsang (ed.), *Stem Cell Biology and Regenerative Medicine in Ophthalmology*,
Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-5493-9_6,

among researchers. Although typically considered a distinct population of stem cells, hemangioblasts that arise from HSCs may diffentiate into EPCs [6]. Growing evidence suggests a role for stem cells from bone marrow, especially EPCs, in neovascularization in pathologic conditions. Given the complexity of the origins of these various stem cells, the broad, simplistic term bone marrow stem cells (BMSCs) will be used. Herein, the role of BMSCs in response to pathologic conditions and the potential therapeutic use of these cells in the treatment of retinal disease are discussed.

BMSCs in Tissue Injury

Revascularization is a critical step in repair of ischemic tissues and organs. After embryonic organogenesis is complete, further angiogenesis typically occurs only in pathologic situations as a result of the production of pro-angiogenic factors and increased permissiveness in the host endothelial cell environment for neoangiogenesis. In response to upregulation of angiogenic factors, most notably vascular endothelial growth factor (VEGF)-A [7], BMSCs are mobilized into the peripheral circulation [8, 9]. EPCs are thought to play a critical role in this process since they can differentiate into the structural elements of new blood vessels. However, in some organ systems, HSCs may also play an important role in recruitment and homing of EPCs to the target [10].

Cells of bone marrow origin have been shown to migrate to the site of tissue injury in a number of organ systems. A common experimental model to verify the bone marrow origin of these cells is to create stable chimeric mice, which have been irradiated to destroy all host bone marrow cells then transplanted with exogenous, labeled BMSCs. Using such a paradigm, bone marrow-derived cells have been shown to contribute to granulation tissue [11] and tumor angiogenesis and to revascularize and improve function in hindlimb ischemia [12].

Bone marrow-derived cells have also been shown to migrate to sites of injury in the eye. In irradiated mice stably reconstituted with Sca-1+/c-Kit+/lin− cells, bone marrow-derived cells were found in retinal neovascularization caused by photocoagulation [13]. In a similar experiment model, labeled bone marrow cells migrated to the site of RPE injury caused by mechanical disruption of Bruch's membrane with injection of an adenoviral vector carrying the VEGF-A gene or chemical injury with sodium iodate [14].

Therapeutic Potential for BMSC Transplantation

Acute transplantation of BMSCs to improve revascularization has been applied to models of ischemic disease in different organ systems. Asahara et al. [7] demonstrated that putative endothelial cell precursors (CD34+/Flk-1+) isolated

from the peripheral blood of adult humans improved revascularization in a rodent model hindlimb ischemia. Transplantation of bone marrow-derived cells has also been shown to improve function and survival of ischemic myocardial cells [15, 16]. In a stroke model, bone marrow-derived stem cells improved angiogenesis in the central nervous system [17, 18].

From a technical perspective, BMSCs have several advantages over other types of stem cells in the setting of therapeutic transplantation. First, BMSCs can be readily isolated from peripheral blood of adults, without the ethical concerns and practical limitations of embryonic stem cells. Second, autologous transplantation may be possible, obviating the need for immune suppression to prevent graft rejection and eliminating the risk of inadvertent exposure to undetected pathogens in the transplanted material. Third, they do not require any genetic manipulation to maintain their pluripotency, unlike induced-pluripotent stem cells.

BMSC Transplantation for Retinal Disease

Ischemic retinal diseases such as diabetic retinopathy (see Fig. 6.1), retinal vein occlusion, retinal artery occlusion, and retinopathy of prematurity, are rational candidates for therapeutic BMSC transplantation. Current treatment strategies to prevent severe vision loss from ischemic retinal disease include panretinal photocoagulation, which decreases the production of angiogenic factors such as VEGF by ablating ischemic peripheral retina [19] (see Fig. 6.2) and anti-VEGF intravitreal pharmacotherapy, which temporarily neutralizes VEGF [20]. Unlike these current therapies, revascularization of ischemic retina with therapeutic angiogenesis could prevent sequelae of severe ischemic retinopathy and potentially improve retinal function in a durable, nondestructive manner.

Caballero et al. [21] examined the effects of intraocular transplantation of human CD34+ cells in rodent models of ischemic retinal disease, including neonatal hyperoxia-induced retinopathy, retinal ischemia-reperfusion in adult mice (temporary central retinal artery occlusion induced by ophthalmic hypertension) and streptozocin-induced diabetes in adult rats. Transplanted cells co-localized with damaged host vasculature in all three models and vessel caliber and patency was improved in transplanted eyes compared to controls. Interestingly, there was no qualitative difference between intraocular and systemic route of administration, consistent with the ability of these cells to migrate to sites of tissue injury from peripheral blood.

Interestingly, BMSC transplantation may have applications in non-ischemic retinal disease. Otani et al. [22, 23] studied the effects of intravitreal injection of BMSCs in two models of inherited retinal degeneration, the *rd1* mouse and the *rd10* mouse. In both models, bone marrow-derived Lin− cells improved retinal thickness, retinal vascular density, and retinal function as measured by ERG. These findings suggest that transplantation of these cells may have effects not directly attributable to angiogenesis. In support of this hypothesis is the finding that

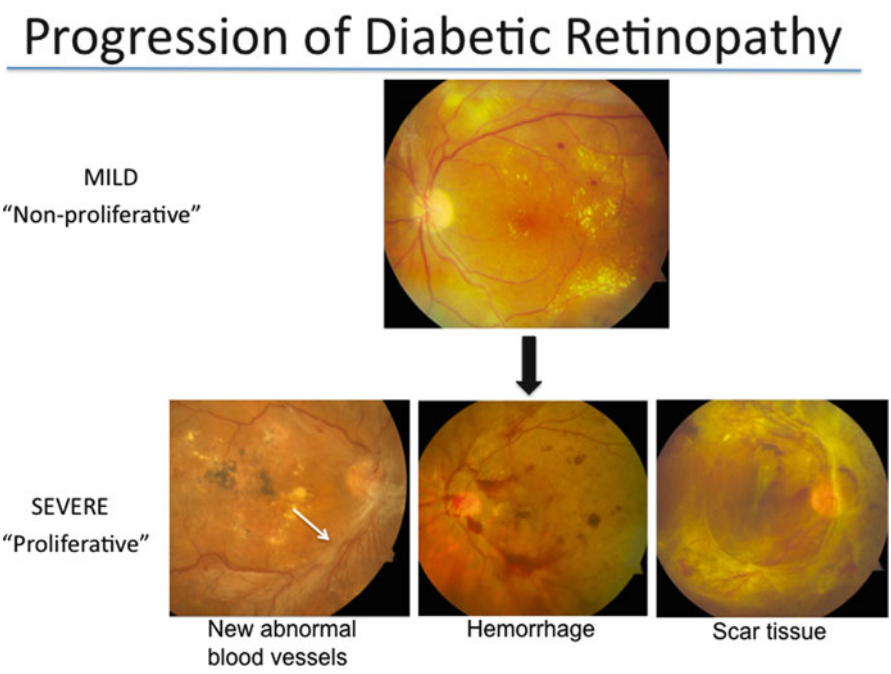

Fig. 6.1 Diabetes is a vascular disease that can obliterate the capillary bed in the retina. Diabetic retinopathy begins with hemorrhages in the blood vessels around the retina (pictured above as red spots in the mild, or non-proliferative stage). The retinal tissue responds to ischemia by producing a high level of vascular endothelial growth factor (VEGF). Following this, abnormal blood vessels begin to form (white arrow, bottom left), a process called neovascularization. The new vessels are unusually fragile and may produce further hemorrhaging (bottom center), a precursor to widespread scarring (bottom right). Ultimately, diabetic retinopathy may lead to retinal detachment

BMSC-derived cells found at the site of RPE damage differentiate into RPE cells [14]. Genetic analysis of these cells suggest that this is not the result of fusion of bone marrow-derived cells with unlabeled, differentiated host cells.

If BMSCs capable of mitigating damage from eye injury already exist in the body normally, why would therapeutic transplantation be beneficial? One simple rationale is that the normal reparative response is limited by the number of available BMSCs. Since BMSCs can be expanded ex vivo, greater numbers of these cells may augment the normal host response [24]. A second rationale is that certain pathologic settings, such as diabetes, could affect the availability or ability of BMSCs to participate in restorative anigogenesis. Fewer HSCs are found in the peripheral blood of diabetic human subjects [25, 26] and in rats with experimental diabetes [27], suggesting that fewer of these stem cells may be available to participate in injury response in peripheral tissues. Hyperglycemia has been shown to impair survival and function of circulating blood-derived progenitor cells in vitro [28]. Further, transplantation of CD34+ cells isolated from diabetic subjects did not show beneficial effects seen with cells from nondiabetic subjects in rodent models of ischemic retinopathy [21].

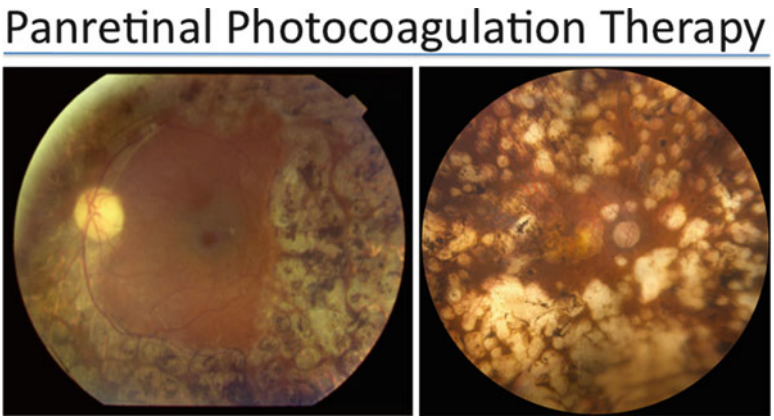

Fig. 6.2 Panretinal photocoagulation has been successful in treating diabetic retinopathy. Through this laser treatment, the ischemic retina is ablated and the production of vascular endothelial growth factor (VEGF) is slowed. The mechanism for this treatment involves 1200 to 1600 tiny laser burns scattered throughout the outer retina, eliminating photoreceptors and RPE. After panretinal photocoagulation, areas of inner retina formerly deprived of oxygen due to the poor perfusion of inner retinal vessels begin to receive more oxygen. A decreased number of hypoxic cells decreases VEGF, halting neovascularization. The macula, or the center of the retina, is typically not treated with laser (right), but in some cases, laser may also be applied to this area (left)

Conclusion

Recent advances in our understanding of the role of BMSCs in neovascularization have refined our understanding of the response to tissue injury and laid the foundation for BMSC-based therapeutic angiogenesis. Furthering our knowledge of the roles of specific subsets of BMSCs in angiogenesis and the factors that regulate the process will be critical to further development of this therapeutic approach. BMSC-based therapy holds promise for the treatment of ischemic retinal disease, but special consideration must be given to the potentially deleterious effects of pathologic neovascularization seen in these diseases. Future work will hopefully help improve our understanding of and ability to manipulate differences between pathologic and therapeutic angiogenesis in retinal disease.

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Chapter 7
Stem Cells, Mechanism-Based Therapies and Regenerative Medicine Approaches

Xining He, Deniz Erol, and Stephen H. Tsang

Abstract The degeneration of photoreceptors and retinal pigment epithelium is the cause of many inherited and acquired ocular disorders that can lead to blindness. Some of these disorders are caused by single gene mutations while others are caused by polygenic mutations or environmental factors. In the past, animal models and gene therapy have aimed at correcting single gene mutations in diseased retina. But more recently, advances in stem cell research, together with advances in retinal tissue transplantation, have moved forward the possibility of treating patients with polygenic or environmental causes of retinal degeneration. Stem cells can now be derived from many sources, including peripheral blood, and successfully differentiated into retinal precursors. In addition, retinal tissue transplantation has been successfully demonstrated in both animals and humans. This article will review recent advances in the study and treatment of retinal disease.

Introduction

The outer layers of the retina—the photoreceptors and retinal pigment epithelium—are preferentially affected in age-related macular degeneration (AMD), Stargardt disease, retinitis pigmentosa (RP), and Leber congenital amaurosis. These diseases

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S.H. Tsang (ed.), *Stem Cell Biology and Regenerative Medicine in Ophthalmology*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-5493-9_7,

affect a significant number of people around the world. Current therapy is aimed at slowing the progression of these diseases, but patients inevitably experience blindness. A cure for these retinal degenerative diseases would require the production and successful transplantation of retinal cells into patients with retinal degeneration.

Anatomy

The retina is comprised of ten layers, from the retinal pigment epithelium (RPE) to the inner limiting membrane, that convert light reception to nerve signals. The outermost layer of the retina contains the RPE, which supports photoreceptors. The external limiting membrane separates the photoreceptors from their cell bodies in the outer nuclear layer. The outer plexiform layer contains the synapses of photoreceptors with bipolar cells. Bipolar cell bodies are located in the inner nuclear layer. In the inner plexiform layer, bipolar cells synpase with ganglion and amacrine cells. Ganglion cell nuclei are located in the ganglion cell layer. Ganglion cell axons are located in the nerve fiber layer. Finally, the inner limiting membrane contains the Müller cell footplates. The photoreceptors and RPE are preferentially affected in both inherited and non-inherited forms of retinal degeneration.

The RPE performs a number of functions to support photoreceptors, including light absorption, nutrient transport, and the phagocytosis of damaged photoreceptor outer segments. The impairment of phagocytosis, as demonstrated in Royal College of Surgeons (RCS) rats, leads to photoreceptor death [1]. The RPE also contains the isomerase RPE65, which converts all-*trans* retinal to 11-*cis* retinal. A defect in RPE65, such as that found in Leber congenital amaurosis type 2, also causes photoreceptor degeneration. Photoreceptors are adjacent to the RPE and are composed of inner and outer segments. The outer segments contain rhodopsin, a photosensitive pigment that isomerizes 11-*cis* retinal to all-*trans* retinal in the presence of light. The RPE is supported on the side adjacent to the capillaries by Bruch membrane, which controls the movement of molecules across the epithelium. In inherited and acquired retinal degeneration, the loss of photoreceptors, through defects in the RPE or otherwise, can lead to blindness.

Mechanism of Disease

Age-related macular degeneration is the leading cause of blindness in the developed world in people ages 60 and older (see Fig. 7.1). In the early stages of AMD, the macula is characterized by the accumulation of lipofuscin within the RPE cells and deposits of drusen between the RPE and the Bruch membrane. Drusen disrupts the RPE from the Bruch membrane and leads to a host of oxidative and inflammatory responses that ultimately result in RPE death and central vision loss (see Fig. 7.2). In advanced stages of AMD, the macula is characterized by geographic atrophy, sub-RPE deposits and choroidal neovascularization.

Although AMD is not a hereditary macular dystrophy, several susceptibility genes have been identified [2–5]. Complement factor H (*CHF* Y402H haplotype),

Fig. 7.1 Macular degeneration is caused by degeneration of the retina resulting in vision loss in the macula at the center of the visual field. As this simulated photographic representation shows, macular degeneration makes it difficult to focus on central objects and recognize faces. However, the condition leaves peripheral vision intact

which inhibits the complement cascade from reacting against the body's own tissues, is a major susceptibility gene in AMD [2]. The SERPING1 gene product and polymorphisms of the *SERPING1* gene, which regulate the classical complement pathway, are also associated with AMD [3]. Genes associated with oxidative stress have also been implicated. The *LOC387715*/*ARMS2* gene on chromosome 10q26, which produces a protein found in the mitochondrial outer membrane, is the second major susceptibility gene identified in AMD [4, 5]. Together, *CHF* Y402H, *SERPING1*, and *LOC387715*/*ARMS2* are found in over 60 % of cases of AMD. Although genome-wide association studies have identified a number of genes,

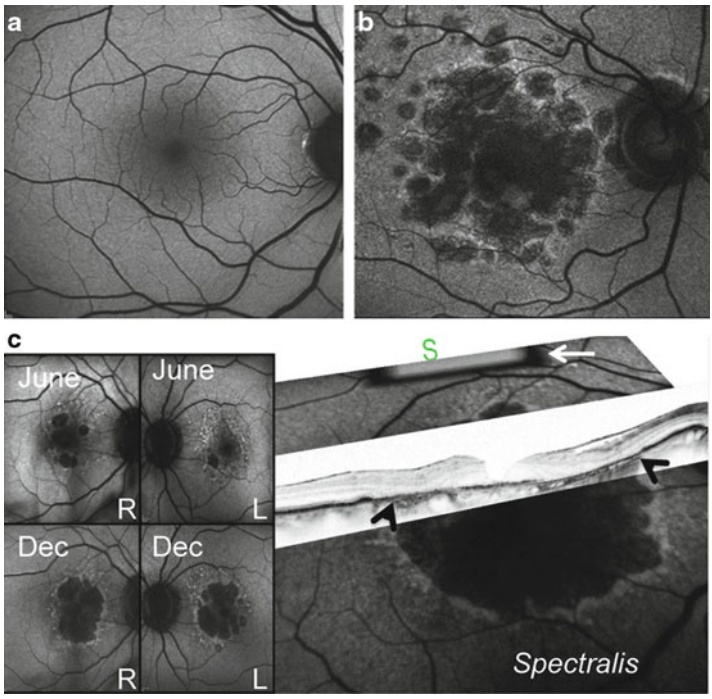

Fig. 7.2 Retinal cell loss in macular degeneration. (**a**) Normal fundi from a healthy subject. (**b**) Fundi from a patient with advanced macular degeneration showing RPE loss. (**c**) A2E autofluorescence (AF) images (over 18 months, top to bottom) of non-exudate age-related macular degeneration, showing progressive RPE loss. This patient is double-homozygous for *ARMS2* (T-in/del), *HTRA1* (**a**), and *CFH* (402Y) and non-cGMP grade iPS has already been generated. In 2007, visual acuity was 20/40 in right (RE) and 20/25 left eye (LE). In 2011, her vision was 20/400 (RE) and 20/400 and the patient lost her independence in activities of daily living. Scattered, nonconfluent drusen are visible at the posterior pole, along with minor pigmentary alterations. Expanding spots of RPE loss can be seen in the area of increased AF nasal and superior to the large central spot of atrophy. A higher AF signal indicates excessive amounts of lipofuscin in the retinal sites that will continue to undergo RPE death, leading to absolute scotoma (areas of vision loss). *White arrow* indicates the internal fluorescence reference *rectangle*, which is mounted in the intermediate retinal plane of the camera. The reference is in focus with the image and can account for variable laser power and detector sensitivity during image analysis. *White arrows* on optical coherence tomography (OCT) mark the loss of RPE. *S*, superior macular region

including those described above, that are highly associated with AMD, the underlying molecular mechanisms that ultimately lead to RPE loss are not known. The mechanisms that lead to the cells' disappearance are likely related to a mixture of both hereditary and environmental factors.

The photoreceptor/RPE complex is also preferentially affected in Stargardt disease, the most prevalent of the hereditary macular dystrophies. Stargardt disease leads to a progressive loss of central visual acuity in the first two decades of life. *ABCA4* is a gene found to be mutated in 70 % of Stargardt cases [6]. *ABCA4* encodes for a protein called Rim, located in the rims of photoreceptor disc membranes, which is involved in the transportation of a vitamin A intermediate to the RPE, where it

prevents lipofuscin from accumulating [7]. Lipofuscin accumulation results from the incomplete digestion of phagocytosed outer segment photoreceptors in the RPE. A major component of RPE lipofuscin is A2E, a by-product of the visual cycle [8]. *ABCA4* mice, an animal model for Stargardt disease, develop an accelerated accumulation of A2E in the RPE, thickening of the Bruch membrane, and visual loss [9]. Clinically, Stargardt disease results in lipofuscin accumulation in the RPE and photoreceptor inner segments, RPE and choroidal vascular atrophy, macular photoreceptor loss, and reactive Müller glial hypertrophy. A retrovirus carrying the wild-type *ABCA4* gene was found to reduce lipofuscin accumulation in a mouse model of Stargardt disease [10].

Retinitis pigmentosa (RP) is a heterogeneous group of disorders characterized by a progressive deterioration of rod and cone photoreceptor function (see Fig. 7.3). Currently, nearly 250 different genes and mapped loci have been found to be implicated in RP, which is the leading cause of inherited blindness in children and young adults. One form of retinitis pigmentosa, Leber congenital amaurosis, is a rare but severe inherited rod-cone dystrophy affecting children within the first year of life. More than a dozen genes have been implicated in the condition. For instance, RPE65, implicated in Leber congenital amaurosis type 2, codes for a protein found in the RPE that recycles the by-product of the visual cycle all-*trans*-retinal back to 11-*cis*-retinal. Gene therapy introducing RPE65 has restored retinal function to Briard dogs that have a mutated version of the *RPE65* gene [11]. Human trials have attempted to restore sight in Leber congental amaurosis using the same viral vectors to introduce the *RPE65* gene to study subjects, but have demonstrated mixed results [12].

To treat polygenic diseases such as AMD, gene therapy would need to identify a number of genes and be able to reintroduce them back to the patient's genome effectively. Moreover, gene therapy is not effective in advanced stages of retinal degeneration after the target cells have degenerated. Therefore, developing the means to produce young cells that can replace aged cells has become an important goal for stem cell research in the retina.

Embryonic Stem Cells and Retinal Cells

Stem cells have the potential to differentiate into a number of cell types and are capable of extended self-renewal. There are several possible sources from which the cells can be derived. For instance, embryonic stem (ES) cells are stem cells derived from the inner cell mass of blastocyst-stage embryos. They consist of three germ cell layers, endoderm, mesoderm, and ectoderm, each with the potential to differentiate into a variety of cells.

In 2004, the Takahashi group described the differentiation of monkey ES cells into RPE by culturing ES cells with PA6 stromal cells in a differentiating medium [13]. The resultant ES cell-derived pigment epithelium expressed typical RPE markers and enhanced the survival of photoreceptors when grafted into the subretinal space of 4-week-old Royal College of Surgeons rats [13]. The generation of photoreceptors from mouse ES cells was more complicated, requiring culture

Fig. 7.3 In contrast with macular degeneration, retinitis pigmentosa causes narrowing of peripheral vision until the visual field is eliminated. For advanced RP patients, objects are seen as if through a keyhole. Two images simulate the vision of an RP patient

under serum-free suspension conditions with Wnt and Nodal antagonists (Dkk1and LeftyA), then activin and serum (termed SFEB/DLFA-treated ES cells) [14]. These SFEB/DLFA-treated ES cells expressed Rx and Pax6, suggesting photoreceptor differentiation. They were able to integrate into explanted embryonic retinal tissue; approximately 10 % localized to the outer nuclear layer and possessed a protrusion on the outer side characteristic of photoreceptor outer segments [14].

Table 7.1 Diverse sources of stem cells: comparing research promise and challenges

Cord blood/bone marrow stem cells	Advanced cell technology's embryonic stem cells trial at UCLA	Adult skin-derived stem cells (iPS)
FDA approval	FDA approved safety trial showed promising results from one Stargardt and one macular degeneration patient. Vision was improved in both patients within a 4-month period Results from efficacy trial expected by 2015	In development
No immunosuppression, stem cell survival is transient	Immunosuppression similar to a kidney transplant regimen is required	No immunosuppression needed for adult skin derived stem cells since they are derived from patient
No gene correction in stem cell necessary	No gene correction in stem cell necessary	Gene correction in stem cells may be necessary in many cases

More recently, several groups have described the generation of RPE and photoreceptors from human ES cells [15–19]. Vugler et al. [17] described the generation of RPE cells from human ES cells. The human ES-derived RPE cells were shown to express markers of developing RPE such as OTX1/2 and Pax6, as well as markers of mature RPE such as RPE65 [17]. When transplanted into the subretinal space of RCS rats, the cells maintained the expression of RPE65, while downregulating the expression of developing markers [17]. The same human ES-derived RPE cells were found capable of phagocytosing porcine outer segments *in vitro* and human photoreceptors in an artificial *ex vivo* human retina system [18]. Osakada et al. [19], also in 2008, generated RPE and photoreceptors from human ES cells by culturing human ES cells with Wnt and Notch inhibitors to produce RPE, and then adding retinoic acid and taurine to generate photoreceptors.

ES cells carry a risk of immune rejection because they do not have the same immune profile as the host. There are also ethical issues associated with the use of ES cells. Other potential sources of stem cells include fetal stem cells and adult stem cells. Fetal stem cells can be used for transplantation when they are allowed to differentiate into retinal cells but transplanted before they form any intrinsic connections. However, ethical issues are associated with fetal stem cells as well, and the supply of fetal stem cells is limited. Adult bone marrow and umbilical cord blood also contain stem cells capable of differentiating into retinal cells. The benefit of using cord blood or bone marrow stem cells is that they make immunosuppression after transplantation unnecessary (see Table 7.1). In addition, no gene correction is needed because these cells do not contain the culprit genetic mutations. An FDA approved safety trial at the Wills Eye Institute is currently underway to test for the use of cord blood or bone marrow stem cells in the treatment of macular disease.

Induced Pluripotent Stem Cells and Retinal Cells

The use of ES cells in transplantation is limited by the possibility of immune rejection and other ethical issues. The development of induced pluripotent stem cells in 2006 has provided a way of producing pluripotent stem cells from somatic cells without the use of an oocyte, thereby avoiding immune rejection [20]. Takahashi and Yamanaka were able to induce pluripotency in somatic cells by the ectopic expression of four defined transcription factors: *Oct4, Klf4, Sox2,* and *Myc* [21, 22]. The most common source of somatic cells has been skin fibroblasts, but the risk of complications from using skin fibroblasts has led to the search for other sources of patient tissue [20].

More recently, induced pluripotent stem cells (iPSCs) have been derived from terminally differentiated T cells in the peripheral blood [23–25]. During this process, mononuclear blood cells were isolated and induced by integrating and non-integrating vectors such as lentiviruses, retroviruses, or temperature-sensitive mutated Sendai viruses into embryoid bodies containing endodermal, mesodermal, and ectodermal markers [23–25]. When injected subcutaneously into mice, the cells developed into tumors containing tissue from all three germ cell layers [23–25].

Clearly, the use of integrating vectors increases the potential for tumor formation and mutagenesis in the transplant's host. Any cells treated with genes and vectors and then transplanted into human patients need to be assessed for tumorigenesis beforehand. In 2009, Hirami et al. [26] described the generation of human photoreceptors and RPE cells from iPSCs using Wnt and Notch inhibitors to produce RPE, and adding retinoic acid and taurine to generate photoreceptors. The use of iPSC-derived retinal cells brings us closer to true personalized medicine. Unfortunately, tumor formation is a risk of both ES cell and iPSC transplantation because not all stem cells differentiate using a differentiation protocol, and the non-differentiated cells are transplanted along with differentiated cells into the host retina, where the results are unpredictable.

Stem Cell Transplantation

Stem cell transplantation has the potential to provide treatments for the advanced stages of retinal degeneration with retinal neuron loss. However, for the transplant to succeed, the transplanted cells must integrate into the host retina through synapse formation. Photoreceptors should be able to integrate more readily than other types of neurons; they only need to create one synaptic connection with a second-order neuron, since their afferent responses depend not on receiving impulses from other neurons but from light. Meanwhile, RPE does not require reconnection with another neuron, although it does require integration with the Bruch membrane. Interestingly, grafted RPE do not require contact with the Bruch membrane in order to restore retinal function in animal models [27]. Moreover, a few transplanted cells can restore visual function globally.

Early transplantation experiments in rodents using whole sheets of fetal retina demonstrated that the transplanted cells only integrated into the host retina to a limited extent. The increase in visual function was thought to be largely due to the expression of trophic factors by grafted tissues that maintained survival of existing host photoreceptors. In 1999, human fetal neural retina tissue was transplanted into six patients with RP and four patients with AMD [28]. Visual acuity improved in 70 % of the patients. One patient maintained his improved visual acuity for 6 years. More recent studies of transplanted human fetal retinal tissue to patients with RP have not shown improvement in overall visual acuity [29]. Transplantation of fetal retinal tissue into patients with advanced AMD has even demonstrated graft rejection [30–32].

In contrast to fetal retina tissue transplantation, stem cell transplantation may have better integration potential in the host retina. Stem cells have greater plasticity and migrating capacity. A rat model of RP has shown rescue by a human RPE cell line (ARPE-19) and by RPE cells derived from human ES cells [33–36]. A human RPE cell-line (ARPE-19) improved retinal function in RCS rats [34, 35]. Similarly, human stem cell-derived RPE also improved visual function in RCS rats [17, 33, 36]. Most recently, iPSC-derived RPE improved retinal function in RCS rats [18]. In 2010, RPE derived from ES cells of C57BL/6J-*Tyr*$^{c\text{-}2j}$/J (C2J) mice were transplanted into a spontaneous mouse mutant model of RP, termed the *rd12* mice, on a C57Bl/6J background [37]. The RPE, labeled with yellow fluorescent protein, demonstrated survival of the graft within the host retina (see Fig. 7.4). Approximately one-fourth of the injected mice showed improvements in electroretinogram (ERG) responses after transplantation, demonstrating proper integration of graft tissue and functional rescue [37]. In 2009, photoreceptors derived from human ES cells were successfully transplanted into a mouse model of Leber congenital amaurosis [38]. In this study, transplanted cells integrated into the outer nuclear layer, expressed layer-specific opsin and rhodopsin, and restored the light reflex [38] (see Fig. 7.5).

So far, RPE transplantation into animal models has shown successful rescue of photoreceptors even without integration with the Bruch membrane. This does not appear to be the case in humans [30, 31]. The integration of RPE with Bruch's membrane may be necessary for successful graft transplantation to restore sight in humans. Autologous transplantation of the RPE and choroid has been attempted on patients with advanced forms of AMD. In 2007, a full-thickness patch graft of RPE, Bruch's membrane, and choroid was harvested from the superior equatorial retina and transplanted into the subfoveal space [37]. Graft viability at 6 months was seen in 11 out of 12 patients with good visual outcomes from three patients, although there were surgical complications in eight patients. In a similar study, the autologous peripheral full-thickness graft of RPE, Bruch's membrane, and choroid were positioned under the macula in patients with geographic atrophy and observed for 1 year [39]. Although graft viability was good, surgical complications were high. Five patients required correctional surgery for proliferative vitreoretinopathy. Meanwhile, visual acuity improvement was mixed. Recently, the results of a long-term follow-up study of 133 patients transplanted with RPE and choroid showed that, on average, the vision of the patients had improved up to 7 years after surgery [40].

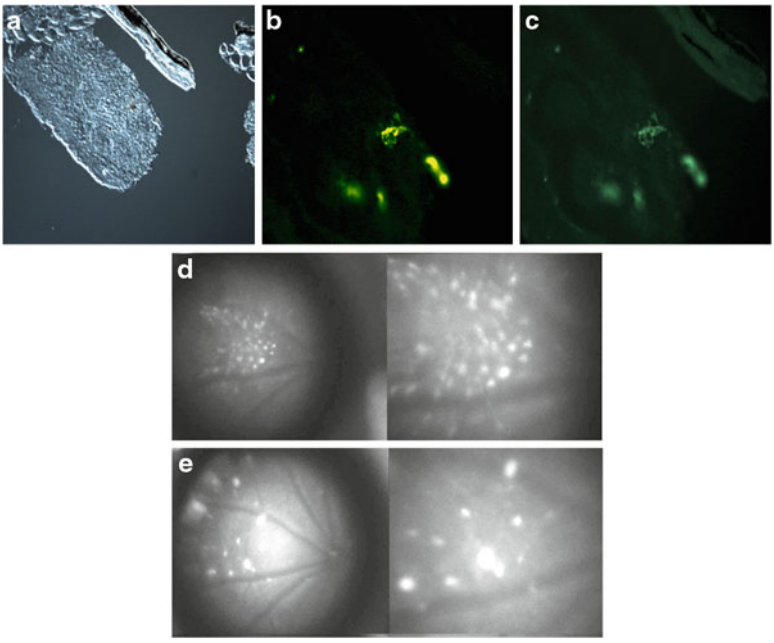

Fig. 7.4 Live imaging of stem cell transplants in animal models of retinitis pigmentosa. (**a**) Representative frozen section of a stem cell transplanted region; (**b**) transplanted stem cells exhibit autofluorescence in the host retina; (**c**) immunohistochemistry using anti-RPE65 antibody showing co-localization of stem cell autofluorescence and RPE65 expression; (**d**, **e**) fundus photograph showing autofluorescence of transplanted stem cells in the subretinal space in a living mouse (**d**) at 9 weeks, and (**e**) at 24 weeks after transplantation

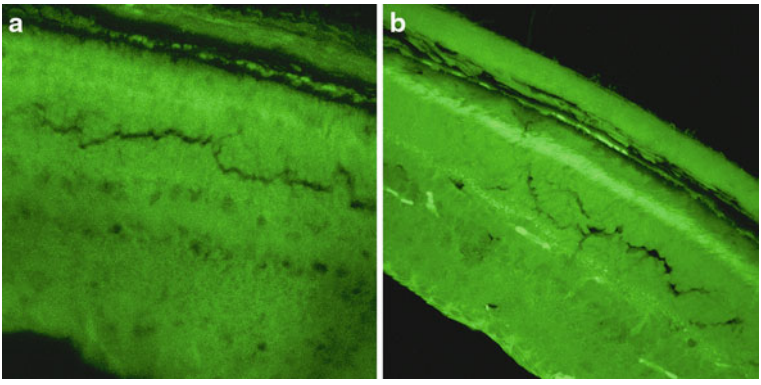

Fig. 7.5 Photoreceptor replacement by stem cells in macular degeneration. A mouse ES cell line engineered with a photoreceptor-specific reporter *Pde6g*::GFP construct fluoresces *bright green* when stem cell-derived photoreceptors are created. (**a**) Control retina; (**b**) fluorescence in the outer nuclear layer (*white arrow*) indicates the formation of new photoreceptors derived from embryonic stem cells

In 2012, a phase I/II clinical trial using human ES cell-derived RPE cells for the treatment of a patient with dry AMD and a patient with Stargardt disease was completed [41]. In both cases, transplanted RPE attached and proliferated in the host retina. At 4 months, the patients' grafts demonstrated neither abnormal growth nor immune rejection. Vision was improved in both patients [41]. Currently, an FDA approved clinical trial is underway to test the safety and efficacy of subretinal transplantation of human ES-derived RPE in patients with Stargardt disease at 12 months. Patients undergoing this trial must take the immunosuppressive drug, cyclosporine. However, no gene correction therapy is necessary because these stem cells are derived from cells that do not carry the mutations responsible for Stargardt disease.

Transplantation with iPSCs carries the risk of reimplanting cells that contain the mutation responsible for the original disease. This is where gene therapy coupled with iPSCs is necessary. For instance, gene therapy in conjunction with iPSCs before transplantation can be used to treat patients with advanced Stargardt disease [42]. However, it is considerably more difficult to correct gene defects in retinal disorders which are polygenic or whose expression is influenced by environmental factors.

Two methods are currently used in the delivery of stem cells into the retina. The intravitreal route uses a small-gauge needle to inject stem cells into the vitreous. This method is easier to perform, but the injected cells have to migrate through the vitreous and inner retina to reach the outer retina. This method cannot direct treatment to a target area, but results in a higher likelihood of the stem cells' survival. Subretinal delivery uses a small-gauge needle to inject stem cells through the sclera and choroid into the subretinal space. This method is more technically difficult to execute, but it provides treatment to a targeted area and has been shown to lead to better differentiation of stem cells.

Conclusion

The goals of current stem cell research in the retina are to be able to obtain cells from patients with retinal degeneration, especially easily accessible ones such as peripheral blood cells, and to use them to generate retinal neurons for transplantation. Using this method, terminally differentiated cells would be induced into pluripotency using a known set of transcription factors and nonintegrating vectors. The set of transcription factors and vectors selected would need to have a low risk of causing tumorigenesis and mutagenesis in the induced cells. Meanwhile, the genetic mutations responsible for the retinal degenerative process would be corrected using gene therapy. Genetically treated iPSCs could then be expanded *in vitro* to the millions of RPE and photoreceptors needed for successful transplantation into the diseased retina. The iPSCs would be allowed to differentiate *in vitro* to the optimal stage for transplantation. Finally, a low-risk procedure would be used to transplant the cells into the subretinal space.

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Chapter 8
New Developments in Retinal Cell Transplantation and the Impact of Stem Cells

Peter Gouras

Abstract Retinal cell transplantation, especially transplantation of retinal epithelium, could provide a method to cure age-related macular degeneration but major hurdles have hampered its advance, such as rejection and surgical technique. The possibility to use autologous fibroblasts from the potential transplant recipient to convert these fibroblasts into pluri-potential cells in culture and then to transform them into retinal epithelium, including checks on their appropriate gene expression offers the possibility of eliminating the hurdle of host graft rejection. A new surgical technique that sections the neural retina for 180° at the temporal ora serrata and folds it nasally to expose the macula and its degenerate epithelial layer can improve the delicate microsurgery. It eliminates jet stream trauma that produces a hole in the equatorial retina and the poor visibility of the epithelium seen through a detached, opaque neural retina. It allows the surgeon to use both hands in removing degenerate epithelium and replacing it with a patch of pristine epithelium. The neural retina can then be folded back to its original location and laser secured at the ora serrata. Transplantation of photoreceptors has greater hurdles, the major one being a guarantee of sufficient synaptic connectivity of transplanted cones to host cone bipolars.

Introduction

The possibility of replacing senescent or defective retinal cells with pristine new ones is an intriguing concept in the field of regenerative medicine. Of all the retinal cells most amenable to transplantation are the retinal pigmented epithelial cells (RPE). These cells form a single monolayer that functions as an independent unit designed to do a number of tasks that affect both the highly specialized

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S.H. Tsang (ed.), *Stem Cell Biology and Regenerative Medicine in Ophthalmology*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-5493-9_8,

photoreceptors and the neural retina. The RPE layer forms the blood/retinal barrier, transports isomers of vitamin A to and from the photoreceptors, ingests and digests the growing tips of the outer segments, and regulates the transport of ions and metabolites to and from the retina. In addition, the RPE synthesizes melanin to reduce the effects of light scatter in the visual image and also counteract oxidative stress. The RPE is post-mitotic with each cell formed at birth continuing to function into old age. This long-term status of a highly active layer of cells leads to senescent changes that compromise optimal function. This affects the macula in particular, probably because of higher energy demands, which is undoubtedly at the root of age-related macular degeneration (AMD), a leading cause of blindness in the elderly. If these senescent cells could be replaced by a youthful epithelium, the defects associated with aging of this epithelial layer could be prevented. In addition diseases that uniquely affect the RPE in younger subjects could also be treated.

RPE Transplantation

RPE transplantation began decades ago facilitated by the ability to dissociate, culture and re-culture RPE cells [1–3]. Culturing not only facilitated transplanting RPE but also allowed labeling the cells in vitro, essential for identifying them in a foreign retina. The first attempt was performed in owl monkeys, primates with a liquid vitreous which can be rapidly removed. An “open sky” procedure was used. The host RPE layer was removed locally in order to put the transplant directly on Bruch’s membrane. This could be done by gently wiping the epithelial layer but detecting this change was impossible at the time of surgery. It was only revealed by postmortem histology. Improvements in optics should allow better visibility of RPE removal. Cultured adult human RPE cells that were dividing in vitro were labeled with tritiated thymidine. The cells were dissociated, sucked into a glass pipette, and slowly injected over the area denuded of host RPE. The monkey’s head was positioned to allow the transplant cells to gravitate toward this area. The eye was closed by suturing the sclera without repairing the retinal incision. Postmortem histology revealed areas of Bruch’s membrane that had been denuded of RPE and other areas where cells resembling cultured human RPE were found. Autoradiography confirmed that the suspected transplants were the tritiated thymidine labeled human RPE. In these early attempts, the neural retina was left detached and with a large retinotomy (Fig. 8.1).

We then sought to reattach the photoreceptors over the transplanted area by working within a bleb detachment of the neural retina in rabbits and monkeys [4]. The bleb detachment was produced by jet stream force from the transplant micropipette. Dissociated, labeled RPE cells were injected over the host RPE. Such transplants survived and phagocytized outer segments. These results prompted us [5] and Turner’s group [6] to use RPE transplantation to treat the Royal College of Surgeons (RCS) strain of rats known to have a defect preventing their RPE from phagocytizing outer segments leading to photoreceptor degeneration.

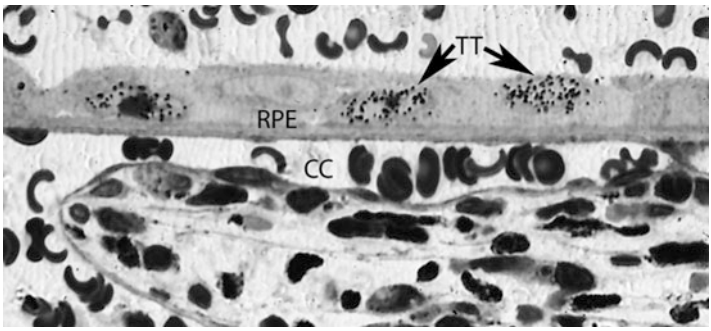

Fig. 8.1 Shows an EM autoradiogram revealing tritiated thymidine grains (TT) present in the nuclei of transplanted human RPE in owl monkey retina several days after surgery

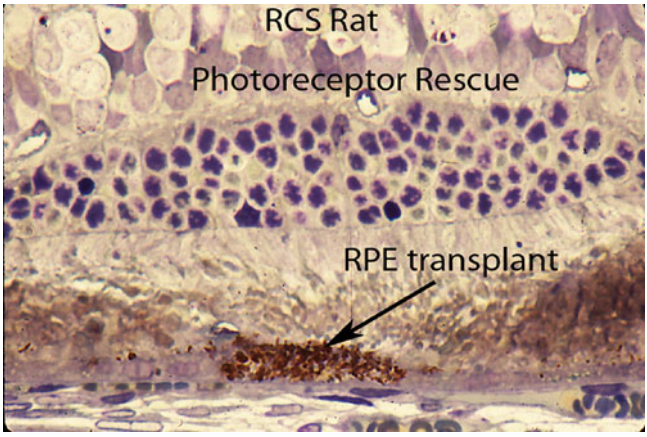

Fig. 8.2 Shows how transplanted normal RPE (*arrow*) can rescue an adjacent group of photoreceptors from degenerating in the RCS rat

Transplantation of normal RPE did prevent this degeneration from occurring in the area where the transplants were located. Electron microscopy revealed that the transplants contained phagosomes and therefore capable of phagocytosis. This result proved that transplantation of RPE could stop the progression of a degenerative retinal disease (Fig. 8.2).

This success prompted the idea that RPE transplantation might have a useful impact on choroidal neovascularization (CNV) that occurs in age-related macular degeneration (AMD). At that time attempts were being made to surgically remove CNV membranes from the macula, but this produced a loss of the adjacent host RPE that was being removed simultaneously. We [7–9] and others [10–12] tried to restore this RPE layer by transplantation after removal of a CNV membrane using either cultured patches of fetal human RPE or dissociated cells. Although some patients maintained foveal function after such surgery, this result was transient lasting less than a month at most (Fig. 8.3).

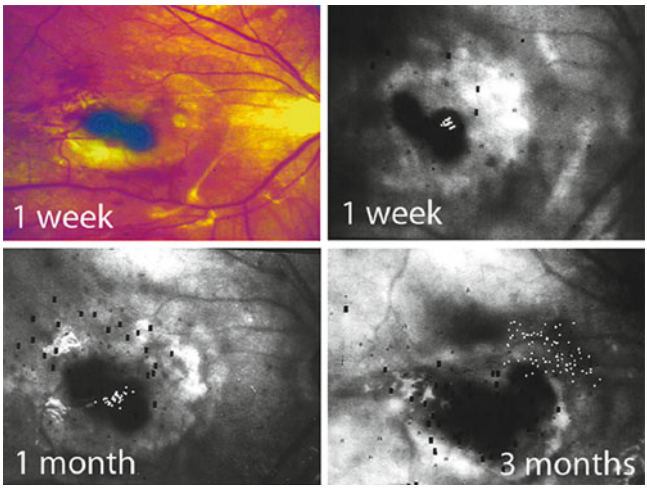

Fig. 8.3 Shows fundus photographs (*upper left*) and scanning laser micro-perimetry (*upper right*) of the macular area at 1 week after transplantation of a fetal human RPE patch following removal of a neovascular membrane. The small white spots show light detection over the fovea, the dark spots show scotomas. The lower photographs show how foveation is lost at 3 months

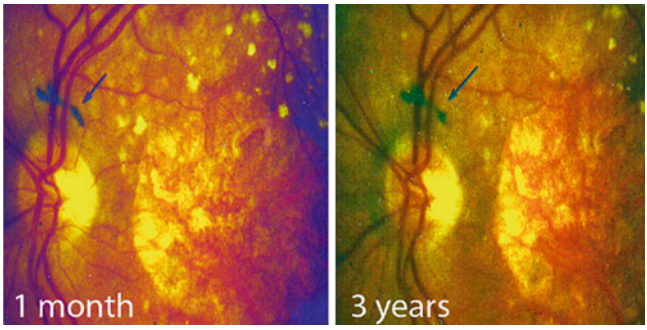

Fig. 8.4 Shows a transplanted patch of heterologous human fetal RPE which floated away after being transplanted to the macula of a patient with geographic atrophy and remained unchanged (*arrows*) for at least 3 years

There was a consensus among those using this methodology that host/graft rejection was destroying the transplant. It is interesting that not all such transplants degenerated, however. Figure 8.4 shows an RPE transplant that slid away from an area of geographic atrophy after transplantation and relocated under a vessel adjacent to the optic nerve where it has remained unchanged for at least 3 years (Fig. 8.4). We have found similar results with human RPE patch transplants to monkey retina. When we transplant a patch to the fovea area versus the peripheral retina, we found a greater chance that a rejection-like picture occurred in foveal transplants (Fig. 8.5). Some RPE xenografts can survive for long periods of time without rejection. We found that foveal transplants are more prone to rejection than peripheral ones [13].

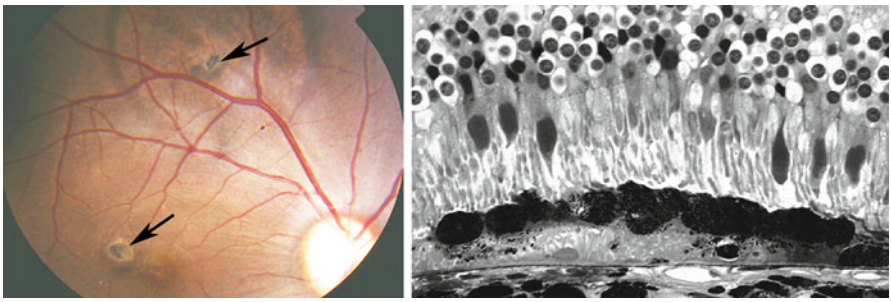

Fig. 8.5 Shows on the *left* a fundus photograph of a rhesus monkey that received two human fetal RPE xenografts, one in the periphery (*upper arrow*) and the other in the fovea (*lower arrow*). The demarcation line of the detachment is larger in the periphery. On the right is an example of such a xenograft, more peripherally located that shows no sign of rejection in monkey retina 5 months after surgery. This transplant and host photoreceptors survive even though the transplant rests on the host RPE layer

Rejection of RPE transplants in the RCS rat has also not been very obvious. There is only one report of host/graft rejection of heterologous RPE transplants in the RCS rat, and this was atypical, being humoral rather than cellular [14]. Therefore the poor success of RPE transplantation may not be due only to rejection but to other factors such as surgical technique.

Autologous RPE Transplants

A new approach emerged that eliminated the problem of host/graft rejection by excising a patch of peripheral RPE together with the choroid from the patient's own retina and transposing this patch to the macular area after a CNV membrane had been surgically removed [15–18]. What is remarkable about this method is that the choroidal vessels in the transplanted patch re-vascularize [19]. But this method has some drawbacks. One is that the host's peripheral RPE patch is senescent and probably less viable than embryonic tissue. The second is that it requires two surgical procedures, the removal of the peripheral RPE patch with its choroid and the macula surgery. Another consideration is the difficulty of working within a macular bleb detachment, which is now being altered by a new surgical technique that exposes the macula.

Exposing the Macula

Surgical manipulation within a bleb detachment is awkward. It restricts the microsurgery, obscures the visibility, and tears the paramacular neural retinal opening needed to enter the bleb detachment. An improvement has been introduced to

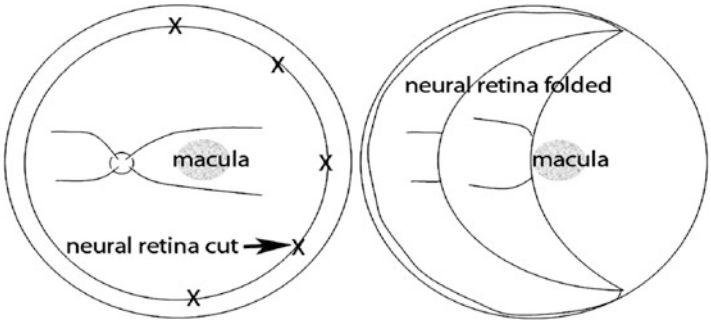

Fig. 8.6 Shows how the neural retina is cut at the ora serrata (crosses) and folded nasally to expose the macula

facilitate such surgery, which allows better access to the RPE by folding the neural retina away from the macula. This approach uses 180^0 retinotomy at the temporal ora serrata in order to fold the neural retina nasally exposing the macular RPE (Fig. 8.6). I have often considered this approach to be advantageous for macular transplantation and surgery. This approach has now been used in human subjects by groups in Italy [20] and China (in press). This allows the surgeon to use both hands in removing a CNV membrane and/or a degenerate RPE layer, in dissecting a peripheral RPE-choroid patch and in placing it properly in the macula.

Removing Degenerate RPE

Reconstructing a new RPE layer should ideally include removing any degenerate RPE cells and if necessary, any CNV membrane. Exposing the macula RPE is important because it allows visualization of the RPE layer directly. With such visibility, the host's macula RPE can be removed more easily as it was using the "open sky" procedure in the owl monkey. Wiping may be more traumatic than using more precisely controlled methods such as an ultra-sonic or a femto-laser probe. Because the femto-laser's pulses are so brief, they do not heat up adjacent tissue keeping their ablation effects localized. Better control of the debridement of the host RPE might be facilitated by robotic surgery where movements of a few microns are possible.

Delivery of the Transplant

Delivering a mono-layer patch of RPE with the proper orientation and flatness presents another problem for transplantation. One method suggested, but not pursued consistently, is encasing a segment of cultured cells in a gel that is rigid

at room temperature but fluid at body temperature. This could facilitate delivering an undamaged, flat transplant with appropriate polarity. If the sclera port were too small to introduce the patch, it could be delivered in separate segments. A supporting scaffold, natural or artificial, may be required to facilitate RPE cell delivery to the eye. Research to improve the biomimetic properties of such materials is being pursued [21–26]. A 3-dimensional scaffold may be effective in growing a 3-dimensional structure such as the entire retina but for therapeutic transplantation a 2-dimensional RPE monolayer seems more appropriate. All manipulations would be facilitated by exposing the macula RPE and Bruch's membrane by folding of the neural retina nasally. Such exposure might even allow transferring an un-encased patch of RPE using a micro-spatula. But reattachment of a folded neural retina is a drawback to the proposed surgery since it is a large detachment including the macula. But if one is attempting to reconstruct a blind fovea, it might be worthwhile.

Iris Pigment Epithelium Transplants

Iris epithelium is closely related to RPE and is readily available by biopsy from potential recipients. But research by several groups [27–29] has not achieved success therapeutically even though it eliminates host/graft rejection. It indicates that there are other factors than rejection affecting epithelial transplantation, such as surgical technique, full exposure and preparation of the site, establishment of the proper flatness of the transplant as a monolayer, the virility of the transplant and its ability to interact in many unique ways with the photoreceptor layer.

RPE Derived from Stem Cells

The concept of using embryonic stem cells to treat disease has been complicated politically because it implied the use and destruction of human fetuses. Embryonic RPE cells have an advantage compared to adult RPE, however, in being very viable in culture and lacking any of the senescent changes that accumulate in adult RPE. Nevertheless they are heterologus and therefore subject to rejection. In 2006 a new era in stem cell research occurred with the demonstration that adult differentiated cells could be induced to become pluripotential by transducing them with unique combinations of transcription factors, *Oct3/4, Sox2, Myc*, and *Klf4* [30], and these pluripotent cells could be further transformed into tissue specific cells such as RPE. This breakthrough meant that human embryos were unnecessary for obtaining stem cells and autologous cells could be obtained from the recipient obviating host/graft rejection although host/graft rejection may still occur [31]. These adult-induced pluripotential cells express similar genes to embryonic stem cells [32]. Recent reports indicate that differentiated adult fibroblasts can be transformed directly

into neural cells by also using a unique combination of transcription factors but without going through a prior pluripotential stage [33–35]. Such transformed fibroblasts can be cultured, transformed into RPE, and tested for the presence of RPE-specific proteins, such as RPE 65, bestrophin 1, CRALP. These transformed cells would be pristine new without the waste products that accumulate in senescent RPE. Such cells could be easily cultured providing the option of genetically engineering them in vitro to express proteins that counteract genetic defects or which are trophic factors that promote survival [36–38].

Prophylactic RPE Transplantation

Will RPE transplantation continue to evolve and become a therapeutic method to treat blinding degenerations such as AMD? Optimists think it can but to do so it has to be performed before there is massive destruction of the photoreceptors in the fovea, as occurs in the late complication of CNV or geographic atrophy. This would then require prophylactic surgery while the patient still has foveal vision, which is the ultimate challenge for this methodology. This cannot be done at present but there is continued research trying to improve it so the method cannot be discarded. What may supersede the simple replacement of degenerate with healthy RPE, however, is the possibility to reconstruct the fovea after total loss of the photoreceptors has occurred by transplanting new photoreceptors, especially cones, together with new RPE. This would allow the reconstruction of the fovea to take place in an already blind eye making any potential failure trivial. This may be the most promising future of cell transplantation in the retina.

Photoreceptor Transplantation

By comparison with RPE transplantation successful transplantation of photoreceptors is much more difficult. But it is sensational since it could restore sight to a blind eye rather than merely saving residual sight, the hope of RPE transplantation. Because it is so sensational it has had a complex history of exaggeration and confabulation. The major difficulty with the approach stems from a key problem, the inability of such transplants to form synapses with host neurons which is essential for proper visual function. There are several reasons for this problem, one obvious and the others arcane. The obvious problem is to obtain photoreceptors devoid of their contacts with their own second order neurons, which block any contacts of the receptors with host second order neurons. The arcane problems involve our inability to control and direct synapse formation from photoreceptors to natural second order retinal neurons.

Whole Retinal Sheets

Many ways have evolved in the many attempts to transplant photoreceptors. One has been to use a sheet of neural retina that is placed between the host RPE layer and the host neural retina [39]. This creates two retinas, one from the transplant and the other from the host, the latter usually with either degenerate or absent photoreceptors. The hope has been that the transplant will extend neural processes that can make synapses with second and/or third order neurons in the degenerate host retina, which could provide a functional connection from transplanted photoreceptors to host ganglion cells and ultimately the brain. Those championing this method have evidence that such synapses can form between the two retinas [40, 41]. Attempts using this method have been tried in both animals and blind human subjects with reports of success. But the approach has not been taken up by the ophthalmic community, undoubtedly because of its relatively minimal effects on vision. The number of synapses that form between these two retinas must not be plentiful enough for any useful vision. This approach seems to be an awkward way to restore retinal function because it does not try to reconstruct the retina in the natural way. Connecting what is the ganglion cell layer of the transplant with the outer nuclear layer of the host retina seems less rational than trying to connect transplanted isolated photoreceptors to their logical second order neurons, bipolar and horizontal cells.

Transplantation of Retinal Micro-aggregates

Small micro-aggregates from mature retina or from 3 to 4 days old mice, an age when photoreceptors are just developing outer segments, have been used as transplants [42–44]. Some of these micro-aggregates contain photoreceptors separated from their second order neurons making them potentially able to form new synaptic contacts with host bipolar cells but such synaptic reconnections have been difficult to find [46]. We have transplanted micro-aggregates into the subretinal space of *rd* mutant mice, at a stage where these mice have lost all of their rods and most of their cones. In early studies we only labeled the donor rods. In later ones both the donor rods and the host rod bipolar cells were labeled [45, 46] (Fig. 8.7). Transplanted, undifferentiated photoreceptors develop normal outer segments which survive for long periods of time, perhaps years, in the degenerate mouse retina. We have learned much from these experiments.

Outer segments only develop if they are oriented in the proper direction, i.e. with the outer segments contacting the RPE layer. Second, the external limiting membrane remains a significant obstacle that blocks contacts between the transplant and host second order neurons. Third and most important we have been unable to detect many synaptic contacts between labeled donor rods and labeled host rod bipolar cells by electron microscopy. Figure 8.8 shows a rare example of lacZ reaction particles labeling a transplanted rod spherule in an adult rd mouse retina in which

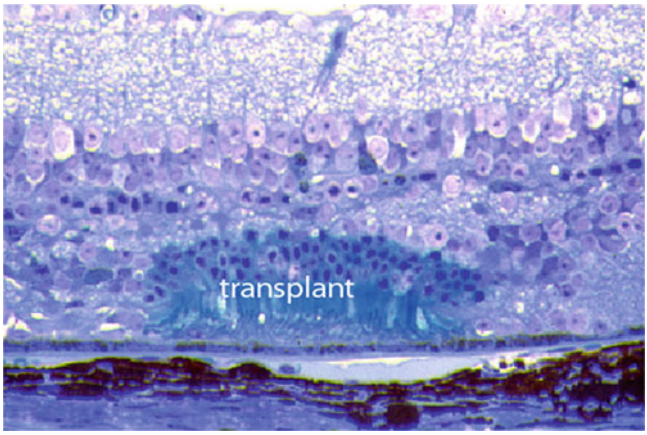

Fig. 8.7 Murine photoreceptors in a micro-aggregate labeled with the lacZ reporter gene (*blue transplant*) and transplanted to the subretinal space of an rd mouse where all of the host rods and most of the cones have degenerated. This transplantation occurred 11 months previously, and there is no evidence of host/graft rejection

only the host rod bipolar cells were also labeled. There is a lacZ particle present on the postsynaptic side of this synapse implying that it belongs to a host rod bipolar cell which suggests that synaptic communication exists between the host and the transplant via a canonical synapse. But this is our only good example among many attempts. We also found membrane-to-membrane contacts between labeled rods and labeled rod bipolar cells, which could allow ephaptic transmission between transplant and host, i.e. K^+ release from the rod could depolarize the host bipolar cell and generate a signal between the transplant and the host retina. Classic synaptic transmission between donor rods and rod bipolar cells that were labeled to be recognizable at the electron microscopic level was extremely rare.

The one shown in Fig. 8.8 is the only convincing sample of such an event we found. The rarity of either canonical synaptic as well as ephaptic contacts between the transplant and host retina indicates that such occurrences are too rare with current techniques. Fourth, we have not seen host/graft rejection, which implies that such neural tissue within the subretinal space may be tolerated or perhaps not detected by the immune system, although there is evidence against such a conclusion [47–49]. It is interesting that the latter study, which transplanted neural progenitor cells from humans into pigs, used laser photocoagulation to promote integration. The rejection encountered might be due to the prior laser treatment that could cause a considerable local inflammatory reaction. It is our impression that rejection is variable and can depend on the local inflammation produced by the surgery. In experiments with subretinal injection of viral solutions that led consistently to cellular rejection, immune-suppression for only a month prevented rejection permanently [50] suggesting that after the initial inflammatory response to the retinal surgery dissipates, the foreign material within the subretinal space may no longer be detected by the immune system.

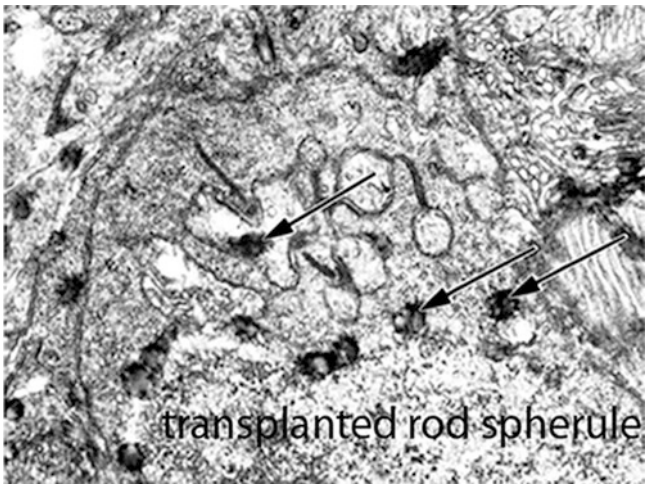

Fig. 8.8 LacZ labeled rod spherule transplanted to the subretina of an rd mouse whose rod bipolar where also labeled by LacZ. The *lower* two arrows indicate Lac Z reaction particles within the spherule. One label (*uppermost arrow*) is in a post-synaptic structure implying that it is a host rod bipolar cell

Transplantation of Dissociated Photoreceptors

Townes-Anderson et al. [51] first reported a method to dissociate isolated rods. We have used this method to isolate photoreceptors for transplantation in rats [52] and mice [42, 43, 52]. Mature as well as progenitor photoreceptors can survive when transplanted to these retinas [53]. Recently cell suspensions of enzymatically dissociated retinas of 1- to 4-day-old mice have been used to obtain isolated photoreceptor cells with similar success [54]. Using a similar approach [55] one group concluded that such transplanted photoreceptors seem to integrate more consistently into the outer nuclear layer and showed more evidence of functional communication between transplant and host retina. The evidence for synaptic mediated function, the key challenge in this field, has been examined by both immunohistochemical and functional methods; the latter including pupillary responses and electrical field potentials from the ganglion cell layer. The ganglion cell recordings indicated increased activity and greater sensitivity in mice with retinal degeneration that had received such transplants than control mice. Curiously, prenatal and later postnatal transplants were less effective. The overall gain in visual function was small, however, most likely because the numbers of integrated and communicating transplanted photoreceptors were few. Attempts are being made to increase the amount of integration by disrupting the blocking outer limiting membrane [56, 57], by enzymatically degrading the inhibitory extracellular matrix (ECM) and cell adhesion molecules, such as CD44 and neurocan [38, 58, 59], by immune suppression [48, 49] by anti-apoptotic treatment of donor cells [38], by enrichment of labeled cells by flow cytometry [60] or by

magnetic-assisted cell sorting [61]. The latter method appears to be the most successful in increasing the integration of young rods enzymatically disassociated from normal murine retina using magnetic beads with antibodies to a surface protein, called cluster of differentiation (CD73) expressed in young rods. This procedure significantly increased the number of transplanted rods integrating into murine retina. This method has the advantage of not requiring genetic modification of the photoreceptors in order to uniquely detect and concentrate them.

Retinal Progenitor Cells

This method involves selecting so-called progenitor cells that exist in the young murine retina especially at the ciliary margin and the optic nerve head [62]. Such cells are undifferentiated, express developmental markers, and can be distinguished by their organization in cultures. Transplantation of such cells into degenerate mouse retina shows that they can express photoreceptor proteins and improve visual performance in behavioral tests of vision. Visual improvement could also be due to trophic influences the transplant exerts on the residual host photoreceptors. This approach has a handicap that the progenitor cells are heterologous and therefore subject to rejection. This method is being eclipsed by recent attempts to transform the host's own differentiated cells into pluripotential cells that should eliminate host/graft rejection.

Transforming Fibroblasts into Photoreceptors

Takahashi and Yamanaka's demonstration [30] that differentiated cells can be reprogrammed into pluripotential cells has had vast confirmation and now involves a variety of techniques [63–77]. This has influenced photoreceptor transplantation [78, 79]. The latter have transformed fibroblasts into pluripotential cells and selected cells that expressed visual proteins for transplantation into the subretinal space of degenerate murine retinas. They obtained integration and expression of photoreceptor genes in these transplants and in addition evidence of visually evoked responses from the mice. This offers the opportunity to take skin biopsies from patients with genetic defects that lead to retinal degenerations and produce photoreceptors that express the deleterious mutation in vitro, which can facilitate studying the pathogenesis of such diseases. The approach is being intensely pursued. It is possible to use only one and not several viral vectors to reprogram these cells [63] or to eliminate the viral vector completely by using nucleo-fection of a polycistronic construct co-expressing *Oct4, Sox2, Klf4*, and *Myc* [80] or only one transcription factor [66].

Cone Versus Rod Photoreceptors

So far most attention has been given to transforming pluripotent cells into rods rather than cones. But for useful vision cones are critical. If one loses all rod function, the handicap is mild with patients only being unable to see in dim illumination; they are not considered blind. If cone vision is lost one is legally blind being unable to read, drive, see colors or recognize faces. It would be important to obtain fine foveal cones that provide us with high resolution vision. One way to obtain embryonic foveal cones is to use human fetal tissue obtained from abortions. Here the fovea and macula can be identified and dissected; the inner layers of the neural retina can be removed from the cone terminals using an excimer laser [81]. Perhaps a less controversial way would be to transform pluripotent cells into fine foveal cones but this is not yet possible. A great experimental advantage of transplanting cones rather than rods is that it would be possible to produce a change in the action spectrum of vision as a result of transplantation, i.e. transplanting ultra-violet sensitive cones into an animal without them would drastically alter the host's vision action spectrum. Using rod transplants the final result can only be based on a stronger or more sensitive response from the animal receiving the transplants which is a quantitative change. Altering the action spectrum of vision would be a stronger qualitative change that would strongly support the conclusion that there was communication of the transplanted cones with the rest of the brain.

Therapy from Photoreceptor Transplantation

It is currently impossible to use photoreceptor transplantation to restore vision in man [82, 83]. Will it ever be? This is a reasonable question to ask because the difficulty in doing this is enormous. The possibility of transforming cells into embryonic cones is on the horizon but the surgical approach to this problem does not exist and is not easy to envision. The major barriers facing the approach are formidable. It requires a way to promote synapse formation from the transplanted photoreceptors and a way to direct them to very specific sites. This is a very difficult problem that may be best pursued by in vitro techniques. An additional problem is being able to facilitate the migration of the cone pedicles to penetrate the external limiting membrane formed by Müller cells, which appears to expand after host photoreceptor degenerate. These are difficult barriers but success with this would be extraordinary.

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Chapter 9
Cancer Stem Cells in Uveal Melanoma

Helen Kalirai, Bertil E. Damato, and Sarah E. Coupland

Abstract Despite extensive research into uveal melanoma many key questions remain unanswered. These include: (a) when during uveal melanoma development does tumour cell dissemination occur? (b) why do uveal melanoma cells preferentially metastasise to the liver? and (c) how do metastatic uveal melanoma cells resist current therapeutic options? One concept that is helping to address similar questions in other cancers is the hypothesis that a subpopulation of tumour cells possesses biological properties akin to those described for normal tissue stem cells. Data suggest that these so-called "cancer stem cells" undergo self-renewal, drive tumour progression and metastasis, and initiate new tumours even after many years of apparent "dormancy", as well as providing a reservoir of cells resistant to chemotherapy. Only now, however, are researchers developing the necessary in vitro assays and in vivo models to evaluate the self-renewal and tumour-propagation capabilities of isolated uveal melanoma cells. This chapter summarises the current concepts of cancer stem cell biology and discusses evidence for the existence of cancer stem cells in uveal melanoma. Particular attention is paid to embryonic gene signatures and developmental signalling pathways, transdifferentiation capabilities and drug efflux transporters, drawing on comparisons with other cancers.

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S.H. Tsang (ed.), *Stem Cell Biology and Regenerative Medicine in Ophthalmology*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-5493-9_9,

Introduction

The concept of cancer stem cells has been the subject of intensive investigation over the last 10–15 years. Such cells are characterised by their relative quiescence as well as the capacity to both self-renew and divide indefinitely. They are likely to be responsible for tumour recurrence and metastatic disease following traditional anti-cancer therapies. Recent studies in uveal melanoma suggest that cells with a cancer stem-cell like phenotype are present in aggressive tumours that are associated with a poor prognosis. It is hoped that insights in this relatively immature field of cancer stem cell research will ultimately create new therapeutic opportunities.

Uveal Melanoma

Uveal melanoma affects around 500 new patients in the UK each year with males and females affected in equal numbers and with the age at presentation peaking at about 60 years [1]. The primary ocular tumour is usually treated by various combinations of radiotherapy, phototherapy and local resection, if possible conserving the eye with useful vision [2]. Enucleation is necessary in about one third of patients.

Despite successful treatment of the primary uveal melanoma, approximately 50% of patients go on to develop metastatic disease usually involving the liver and often many years after their primary treatment [3]. For these patients, the prognosis is poor, with a median survival time of 6–12 months. Metastases from uveal melanoma tend to be resistant to current therapies [4, 5]. Insights provided by the discovery of cancer stem cells have stimulated new research directions in the hope of developing new therapies.

Stem Cells in Normal Adult Tissues and Cancer

Normal adult tissues contain a cell population that is heterogeneous with respect to morphology, function, and gene and protein expression patterns. This tumour cell heterogeneity is believed to reflect the hierarchical organisation of the tissue, with rare self-renewing stem cells able to generate a transient proliferating cell population that undergoes terminal differentiation. These properties are essential for repair and homeostasis in all tissues [6, 7]. Even human tissues traditionally considered stable, undergoing only minimal or slow turnover throughout adult life, are now known to contain specific stem cell populations [8, 9].

Striking similarities between normal tissue homeostasis and cancer have led to the hypothesis that tumours also contain a subpopulation of cells with stem cell-like

properties. It is still unclear, however, whether cancers originate from genetic alterations that have occurred in normal stem cells or whether more differentiated cells undergo oncogenic changes resulting in de-differentiation and the acquisition of stem cell-like characteristics. A recent review discusses the complexities associated with and the distinction between terms such as "tumour initiating cell" and cancer stem cell [10].

Over the last decade, the cancer stem cell theory has been the subject of intense research offering explanations for tumour recurrence following treatment, resistance of tumours to current therapeutic modalities, and the ability of tumour cells to metastasise to distant organs (for detailed reviews see: [11–14]). With many malignancies, including cutaneous melanoma, there is now extensive evidence to support the existence of cancer stem cells [15–19]. Such clues include: the capacity of tumours to show continuous propagation; the ability of a small subpopulation of cells to give rise to tumours in immunocompromised mice that on serial passage resemble the parental tumour; the expression of a range of cell surface markers associated with normal stem cells, e.g. CD44, CD24, CD133; and aberrant activation and involvement of embryonic signalling pathways during tumour progression, e.g. Hedgehog, Wnt and Notch signalling pathways.

Unequivocal evidence however, for the existence of cancer stem cells in solid tumours, such as breast carcinoma and colon cancer, has been difficult to obtain (reviewed in [20]). Many studies have focused on cell surface markers previously shown to be associated with stem cells of other lineages. It should not be assumed, however, that the cancer stem cell markers identified in one tumour tissue are shared with other tumour types.

The isolation of putative cancer stem cell populations using cell surface markers must be accompanied by assays that measure the functional characteristics associated with stem cells, such as self-renewal potential and the capacity to reproduce the cellular heterogeneity of the primary tumour. In vitro assays, which have been used to identify cellular subpopulations with stem cell-like characteristics in other tumour types, include non-adherent sphere assays (originally developed for neural stem cells [21]), serial clonogenic colony forming assays [22], label retention assays [23] and the dye efflux tests [24]. More recent studies have focussed on the relationship between tumour cells and their microenvironment [25–29].

Under normal physiological conditions, the Wnt, Notch, Hedgehog and transforming growth factor (TGF)-β signalling pathways are key players, controlling the balance between self-renewal and differentiation of normal tissue stem cells located in a specialised microenvironment or "niche" [30, 31]. Emerging evidence suggests that in a similar manner to the existence of a "normal stem cell niche", which maintains cells in a stem-like state, cancer stem cells also reside in a particular microenvironment that controls their self-renewal, differentiation and relative sensitivity to cytotoxic insult [11, 32]. Moreover, the microenvironment appears to be of crucial importance for metastasis formation [33]. In solid tumours occurring in breast, colon and lung, similarities between the normal stem cell niche and the tumour microenvironment continue to be revealed [11, 32, 34, 35]. Most solid tumours are composed of heterogeneous cell populations including

mesenchymal stem cells, tumour-associated fibroblasts, endothelial and inflammatory cells, which influence tumour development through complex signalling networks. Increasing evidence suggests that the dysregulation of the Wnt, Notch, Hedgehog and TGF-β signalling pathways promotes cancer stem cell division and hence tumour progression [36]. Elucidation of the complex interplay between the diverse cellular components is of key clinical importance since it may enable the identification of novel therapeutic targets and the development of strategies to target cancer stem cell populations.

Melanocyte Stem Cells, the Neural Crest and Uveal Melanoma

In the adult mouse cornea, neural crest-derived corneal precursors have been identified that express a range of stem cell markers, including Nestin, Notch1, Musashi1 and Slug [37]. In addition, these cells demonstrated remarkable plasticity—i.e. they display the potential to differentiate into cell lineages previously shown to be of neural crest origin, such as myofibroblasts, adipocytes, chondrocytes and neural cells. The developmental origin of uveal melanocytes from cells of the neural crest [38] may suggest that primitive multipotent remnants of these cells are present in the uveal tract. The cancer stem cell concept would then imply that uveal melanoma arises from a malignant transformation of these cells. However, it is also possible that mature differentiated uveal melanocytes undergo de-differentiation and malignant transformation as a result of multiple genetic and epigenetic events, resulting in their progressive development into a cancer cell with "stem cell-like" properties.

The concept of de-differentiation of mature pigmented cells has previously been reported for normal skin-derived melanocytes of the quail [39]. In this study, the melanocytes underwent de-differentiation when clonally cultured in vitro, giving rise to cells that re-expressed genes indicative of an early neural crest lineage (*Sox10*, *FoxD3*, *Pax3* and *Slug*) as well as displaying phenotypic markers of glial and myofibroblastic cells.

Whichever of these theories is correct in terms of the cell of origin for uveal melanoma development, it is becoming clear that there are similarities between uveal melanoma and normal melanocyte development in terms of both the signalling pathways involved and the parallels between metastasis and melanoblast migration from the neural crest during embryogenesis. Studies investigating the cancer stem cell theory in uveal melanoma, however, are lacking. This may be due to the rarity of this tumour type, resulting in an absence of fresh human tissue specimens from which cells can be isolated and their biological properties studied in detail. Nevertheless, data are now beginning to emerge from studies using both uveal melanoma cell lines and primary tumour specimens [40–42].

Experimental Evidence of Cancer Stem-Like Cells in Uveal Melanoma

Primitive Embryonic Gene Signatures in Aggressive Uveal Melanoma

By performing microarray-based, gene expression profiling studies of uveal melanomas, Onken and colleagues have demonstrated that these tumours are clustered into two distinct groups, termed class 1 and class 2 [43]. Class 1 tumours are associated with an excellent prognosis for survival, whereas class 2 tumours are associated with metastatic death as well as histopathological and genetic features predicting this outcome (i.e., epithelioid cell type, looping matrix patterns and chromosome 3 loss). When Onken and associates then searched for functional themes underlying the gene expression classification, they found that the class 2 tumours exhibited transcriptional and phenotypic changes consistent with a loss of melanocytic differentiation and a gain of features associated with a primitive neuroectodermal precursor of the neural crest/melanocyte developmental lineage. To explore this further, these authors compared the gene expression patterns in class 2 uveal melanomas with those of neural, ectodermal, and neural crest stem/progenitor cells using gene set enrichment analysis [40]. They found that class 2 tumours did indeed express genes associated with primitive ectodermal and neural stem cells. In contrast, the class 1 uveal melanoma profiles were consistent with more mature cell types and more highly differentiated melanocytes.

Further evidence of a primitive phenotype in aggressive uveal melanoma cells was reported in a study examining two clonal cell lines derived from a single liver metastasis explant: the aggressive, highly invasive, MUM2B uveal melanoma cell line; and the less invasive MUM2C cell line [44]. In this study, only the aggressive MUM2B cells demonstrated a gene expression profile similar to that seen in pluripotent, embryonic cells.

There is much scope for seeking uveal melanoma cells having active stem cell regulatory networks.

Developmental Signalling Pathways in Uveal Melanoma

The interplay between cancer stem cells and embryonic signalling pathways has been examined in a number of malignancies, including breast, lung and colon. Such studies show that the pathways regulating cell fate during embryonic development also play an important role during tumour progression, invasion and metastasis [36]. Several studies have examined components of these signalling pathways in uveal melanoma.

Expression of Wnt5a, MMP7, and beta-catenin protein was examined using immunohistochemistry in 40 primary uveal melanomas and the results correlated with survival [45]. The proportion of cells immunoreactive for Wnt5a and beta-catenin was significantly higher in tumours from patients with shorter survival, suggesting an involvement of these pathways in tumour progression and metastasis [45].

Cripto 1, a gene member of the EGF-Cripto-1/FRL1/Cryptic family that has been implicated in embryogenesis and in carcinogenesis, has also been examined in uveal melanoma. Cripto-1, together with the TGF-β ligand "Nodal", is a key regulator of embryonic development and is a marker of undifferentiated human embryonic stem cells [46–48]. While Cripto-1 expression is very low in normal adult tissues, it is re-expressed at high levels in several different human tumours, including cutaneous melanoma, modulating cancer cell proliferation, migration and epithelial-to-mesenchymal transition [47, 49]. In formalin-fixed paraffin-embedded uveal melanoma tissue increased Cripto1 protein expression, as assessed by immunohistochemistry, correlated with features associated with enhanced tumour aggressiveness [50].

Understanding the functional role of these pathways in uveal melanoma will require the establishment of model systems that replicate the tumour cells growing in their specialised microenvironment. A few studies in other tumour types are attempting to address this using co-culture techniques and specialised 3D matrices [51–53]; however, very little work in this area is currently being performed in uveal melanoma. Although it remains a challenge across the cancer research community to develop appropriate in vitro models, these will be critical to fully understand the contribution of the signalling pathways that are active in the specialised tumour microenvironment to cancer stem cell behaviour.

Cellular Plasticity in Uveal Melanoma

Cellular plasticity is defined as the ability of cells (usually undifferentiated stem cells) to take on the characteristics of other cell types [54]. In uveal melanoma, there is currently only unpublished data both from our group and that of Harbour and co-workers regarding tumour cell plasticity.

At the 2010 meeting of the Association for Research in Vision and Ophthalmology (ARVO), we reported that cells isolated from a panel of seven primary uveal melanomas variably expressed markers associated with myofibroblasts (α smooth muscle actin), melanocytes (MelanA/HMB45), neuronal cells (βIII tubulin and neurofilament protein) and adipocyte like cells (Oil Red O) when grown in complex medium (Fig. 9.1a) [55]. Furthermore, this increased cellular plasticity was observed predominantly in poor prognosis monosomy 3 uveal melanomas as compared with the expression of only melanocytic markers in good prognosis disomy 3 tumours (Fig. 9.1b).

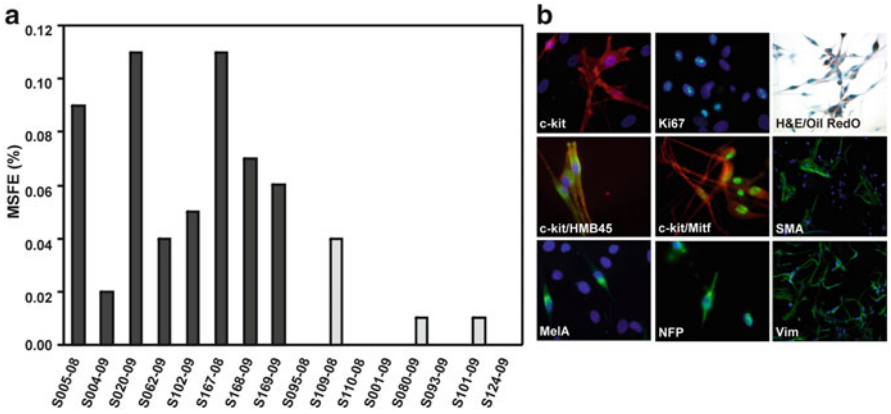

Fig. 9.1 (**a**) Melanomasphere forming efficiency (MSFE) in non-adherent culture. Melanoma cells isolated from 16 primary tumours were grown in non-adherent culture in a complex medium at a density of 2,000 cells/mL. The number of colonies >100 μm was determined after 21 days in culture. The MSFE was determined as: (no. of colonies counted/no. of cells plated) × 100. Cells isolated from monosomy 3 uveal melanomas formed floating spheres in a larger number of cases and at higher efficiency (*dark grey bars*) than cells isolated from disomy 3 uveal melanomas (*light grey bars*). (**b**) Immunofluorescence staining of isolated primary uveal melanoma cells grown in adherent culture. Primary uveal melanoma cells were isolated from tumour tissue and grown in adherent culture using eight well chamber slides for up to 14 days. Cells were fixed with 4% formalin in PBS and indirect immunofluorescence performed with antibodies against c-kit, ki67, HMB45, Mitf, α smooth muscle actin (SMA), MelanA, neural filament protein (NFP) and vimentin (Vim). Some cells were also stained with Oil Red O and haematoxylin. The *panels* show representative immunofluorescence staining of cells from a poor prognosis monosomy 3 tumour and demonstrate expression of markers of several neural crest cell types including myofibroblasts (αSMA), neural cells (NFP) and adipocytes (Oil Red O)

In a review by Harbour et al., reference is made to work showing that class 2 tumours are multi-potent for lineage differentiation but the authors do not provide details of the cell lineages observed [56].

In cutaneous melanoma, Fang and co-workers [16] investigated whether melanoma cells grown in spheroid culture could undergo neural and mesenchymal differentiation. They demonstrated that whilst the cells failed to differentiate into neural lineages, they displayed characteristics associated with cells of mesenchymal lineages (adipogenic, chondrogenic, osteogenic) with varying efficiency. The observation that cells isolated from an advanced primary cutaneous melanoma and a metastatic lymph node underwent mesenchymal lineage differentiation lends further support to the concept of cellular plasticity in aggressive melanomas [18] requiring further investigation in uveal melanoma.

"Vasculogenic mimicry" is another example of tumour cell plasticity and describes the formation of perfusion pathways in tumours by highly invasive and genetically deregulated tumour cells (reviewed in [57]). The formation of loop-like patterns by these vasculogenic structures, which are rich in extracellular matrix, has been associated with poor prognosis in uveal melanoma [58, 59].

Putative Cancer Stem Cell Markers in Uveal Melanoma

Thill et al. demonstrated a variety of putative stem cell markers in uveal melanoma and these included CD133, Pax6, Musashi, nestin, Sox2 and ABCB5; this was done by examining eight uveal melanoma cell lines and eight paraffin embedded primary uveal melanomas, using fluorescence activated cell sorting, immunohistochemistry and reverse transcriptase PCR [42]. A limitation of this study, however, as recognised by the authors themselves, was the inability of these markers to unequivocally identify the cancer stem cell population. Indeed, the expression levels of cancer stem cell markers, such as CD133, CD44 and ABCB transporters, has been highly variable between studies for a particular tumour type and often differs depending on the experimental techniques employed for identification [60, 61].

To overcome these limitations and to characterise downstream molecules, it is necessary to develop standardised functional assays that identify tumour cells with stem cell like properties.

Functional Characterisation of Cancer Stem Cells in Uveal Melanoma

Few investigators have searched for cancer stem cells in uveal melanoma despite the availability of cell lines derived from both primary and metastatic tumours.

Using in vitro clonal analyses, serial passaging, re-plating assays, immunophenotyping and reverse transcriptase-PCR, we recently provided functional evidence that uveal melanoma cell lines contain a subpopulation of self-renewing cancer cells as well as cells that can proliferate and differentiate [41]. In this study, uveal melanoma cell lines showed distinct clonal morphologies in adherent culture akin to holoclones, meroclones and paraclones. These cells formed melanomaspheres (MS) when grown at clonal density in non-adherent culture, which could be serially propagated for several generations. MS demonstrated antigenic heterogeneity expressing markers associated with both a primitive migratory neural crest phenotype (Sox10, Pax3, Notch1 and slug) and a more differentiated phenotype (MelanA and HMB45). Moreover, the uveal melanoma cells surviving cisplatin treatment produced significantly more holoclones than untreated cells, suggesting enrichment for this cancer stem cell-like subpopulation. These data are consistent with studies using cancer cell lines from a range of tumour types, including prostate, breast and head and neck, which have all demonstrated retention of a subpopulation with stem cell-like characteristics [62–65]. Such studies have shown that in vitro clonogenicity correlates well with in vivo tumour initiating abilities [62–64].

Another functional property of the normal stem cell population is their ability to efflux cytotoxic chemicals, thereby protecting them from damage and death. To achieve this, they express high levels of the ABC group of drug transporters

[66, 67]. These same transporters have also been shown to afford protection to cancer stem cells contributing to multidrug resistance [68]. The multidrug resistance proteins, P-glycoprotein (also known as ABCB1), multidrug resistance-associated protein (MRP1) and lung resistance protein (LRP) have been identified in primary uveal melanoma specimens and cell lines [69, 70]. More recently, ABCB1 was shown to be expressed in primary uveal melanomas, but not in normal uveal melanocytes [71]. In the same study, ABCB1− and ABCB1+ cell populations were isolated from the uveal melanoma cell line OCM1A and examined for their tumourigenic and metastatic propensity. The ABCB1+ cells exhibited enhanced clonogenicity and anchorage-independent growth. Furthermore, although both ABCB1+ and ABCB1− cells formed tumours in mice, the ABCB1+ cells did so more efficiently and produced significantly larger tumours than the ABCB1− cells. Similarly, when injected into the tail vein of NOD SCID gamma mice, ABCB1+ cells formed metastatic growths in the liver that tended to be larger and more frequent in number than when the ABCB1− cells were injected. Based on our current knowledge that efflux pumps afford protection to cancer stem cells, shielding them from the adverse effects of therapeutic insult, there is much scope for investigating these proteins and the mechanisms by which they may contribute to therapeutic resistance in uveal melanoma.

Metastatic Uveal Melanoma

Metastasis is a multi-step process involving tumour–stromal interactions that enable tumour angiogenesis, tumour cell migration and invasion into the secondary site. Each type of cancer tends to have its own target organs for metastases. For example, uveal melanomas metastasise preferentially to the liver [72, 73], suggesting that the microenvironment in the secondary organ must be very important, as originally hypothesised by Paget's "seed and soil" theory (reviewed in [74]).

It is well known that following ablation or surgical removal of the primary uveal melanoma, some patients develop metastatic disease after many years of clinical remission [75]. One explanation for this phenomenon of "tumour dormancy" is that the dormant metastatic cells are cancer stem cells in a quiescent state.

Hepatic metastases from uveal melanoma are usually very numerous and widely dispersed throughout the liver. It is not known whether such miliary metastases represents an enhanced cancer stem cell pool in the original primary tumour or de-differentiation of uveal melanoma cells by the local liver microenvironment to a cancer stem cell phenotype.

Conclusions

The molecular characterisation of uveal melanoma stem cells is still at a very early stage. The origin of uveal melanoma stem cells has yet to be determined. Whether uveal melanoma stem cells are derived from melanocyte stem cells, melanocyte progenitors, or more mature melanocytes that have de-differentiated remains unknown and requires further investigation.

Many significant experimental challenges remain. There is a need for standardised and sensitive self-renewal assays, as well as models that mimic uveal melanoma cells and their local microenvironment.

If cancer stem cells are indeed the driving force behind uveal melanoma development, progression and metastasis, this would have profound therapeutic implications. We could at last achieve the breakthroughs that have eluded us for so many years.

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Chapter 10
Current Ex-Vivo Gene Therapy Technologies and Future Developments

Chen-Hsien Su and Deniz Erol

Abstract Ex-vivo gene therapy can entail either the replacement or the addition of genes. In gene addition therapy, a therapeutic gene is inserted directly into the host genome, with the abnormal gene remaining intact. In gene replacement therapy, the genome is modified directly. Homologous recombination technology can be used to perform many of these kinds of gene correction. In the past, gene correction therapy has been hampered by the low efficiency of the recombination event. However, recently engineered zinc finger nucleases (ZFNs) were found to have the ability to successfully stimulate homologous recombination by inducing double-strand breaks at specific DNA sites. Another class of enzyme, the transcription activator-like effector nucleases (TALENs), provides an efficient alternative means to induce specific DNA double-strand at breaks. Meanwhile, newly developed gene correction methods using stem cells and induced pluripotent stem (iPS) cells have made gene therapy more feasible in clinical practice. Cells are taken from patients, harvested, and transformed through induction into stem cells, which have the potential to differentiate into a variety of mature cells types for transplant. Further research is needed to develop gene therapy, which may be used in tandem with embryonic and induced pluripotent stem cell therapy, especially to repair preexisting mutations that may be passed on in iPS cells.

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S.H. Tsang (ed.), *Stem Cell Biology and Regenerative Medicine in Ophthalmology*,
Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-5493-9_10,

Introduction

The gene is the basic physical and functional unit of heredity. It is responsible for encoding all of the information that the body needs to produce proteins. However, when a specific base sequence on a gene becomes altered and begins to produce malfunctioning proteins, genetic disorders can result. In theory, gene therapy can effectively treat these conditions by modifying the specific genes responsible for the disease's development. Ex-vivo gene therapy involves generating stem cells, culturing them, correcting the mutation with viral vectors, and then re-implanting the genetically altered cells into the patient.

Viruses have evolved a pathogenic system for encapsulating and delivering their own genes into host cells. Viral vectors take advantage of the unique properties of viruses that allow them to incorporate into hosts. Among the viral vectors that have been previously used for gene addition, the adeno-associated virus (AAV) has the broad tropism, low immunogenicity, lack of pathogenicity, and viral coding sequences that make it ideal for delivering a vector. AAV has become a widely used and widely preferred gene delivery vehicle, with Food and Drug Administration (FDA) approved trials ongoing

As an alternative to gene addition with viral vectors, recently developed homologous recombination techniques make it possible to cut, edit, and synthesize DNA and otherwise perform gene replacement therapy by excising a faulty gene directly. The homologous recombination used in gene therapy involves targeting a specific mutated strand of DNA (for instance, a mutated gene, or one that causes a genetic disorder) and breaking it off. Next, a therapeutic gene template is delivered into the cells, where it binds to the breakage site. Studies show that targeted and cleanly defined double-strand breaks in the host DNA can be induced by a class of artificial restriction enzymes called the engineered zinc finger nucleases, or ZFNs (Fig. 10.1). These dramatically increase the mutagenesis rate of the targeted genes through homologous recombination. Moreover, compared to ZFNs, another class of artificial restriction enzymes called the transcription activator-like effector nucleases, or TALENs, permit an even higher degree of specificity in creating DNA double-strand breaks. TALENs carry great potential to make homologous recombination a feasible gene therapy technique [1].

To date, gene therapy has primarily been considered as a treatment for mature, differentiated tissues, with an emphasis on replacing the disease-causing genes directly. But the emergence of human-induced pluripotent (iPS) cells has ushered in a new era of possibilities within gene therapy. iPS cells have advantages that are clear even in their name; they are induced from cell cultures, as opposed to derived from embryonic lines, and they are pluripotent, or can become almost any kind of cell. Capitalizing on this ability, stem cell researchers can modify iPS cells through homologous recombination, differentiate them fully, then transplant them back into the host to treat the disease. Preliminary studies of gene therapy on iPS cells have proven successful in animal models. Such ex-vivo gene therapies, while still in development, could become a possible cure for many hereditary conditions.

This review will begin by describing current ex-vivo gene therapy strategies.

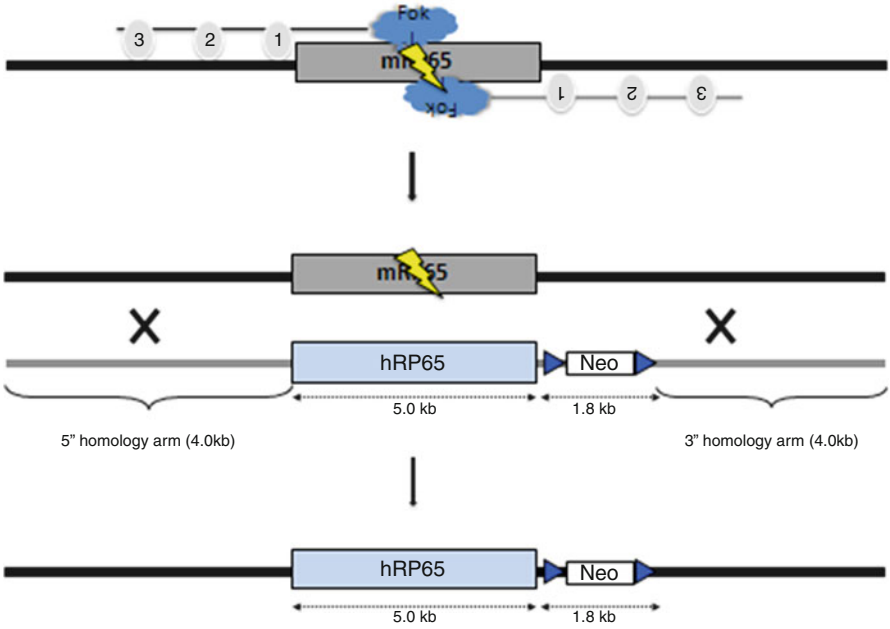

Fig. 10.1 Zinc Finger Nucleases introduce site-specific DNA double strand break and the damaged gene is replaced by homologous recombination containing desired gene and a selective marker

Gene Addition and Replacement: Two Types of Therapies

We propose that the current ex-vivo gene modification therapies can be roughly classified into two categories: the more traditional ex-vivo gene addition therapy and newer ex-vivo gene replacement therapy (also known as gene correction therapy). We propose that the current ex-vivo genemodification therapies can be roughly classified into two categories: the more traditional ex-vivo gene addition therapy and newer ex-vivo gene replacement therapy (also known as gene correction therapy). Both take place primarily outside the body (hence, ex-vivo). To prepare for the insertion of the therapeutic gene, target cells must first be harvested from the patient and maintained in a culture. In gene addition, a viral vector housing a copy of the therapeutic gene is then injected into the cells, where it genetically alters them by the process of transduction. Finally, the genetically modified cells are re-implanted into the patient, where they restore normal functioning of the gene.

A major risk posed by gene addition is the possibility that random portions of the viral genome will become integrated into recipient cells, and that these will make their way into the patient through the transplant or injection procedure. Such random integrations, referred to as insertional mutageneses, would increase the risk of cancer for the patient [2]. The regulation of the therapeutic gene must equally be taken into consideration. The therapeutic gene needs to be expressed by a promoter, a major

region of DNA within the gene that facilitates the transcription and expression of the gene. In general, a strong promoter is used in gene therapy to ensure the therapeutic gene is sufficiently expressed. For instance, a constitutive promoter allows the gene to be continuously transcribed without regulation and without regard to its environment. However, this also poses a danger: the constitutive promoter may go overboard in expressing the promoter and might never stop transcribing new copies, an outcome that would be difficult to regulate in vivo. In contrast, intrinsic promoters, which are currently being tested as an alternative means for promoting gene expression, are well regulated; they can prompt a therapeutic gene to produce certain proteins as necessary but also shut down in the normal environment. In terms of regulation, the intrinsic promoter is preferable for traditional gene therapy.

The process of ex-vivo gene correction therapy is similar to that of gene addition therapy, but involves a different mechanism for transferring the therapeutic gene. In gene correction, the abnormal gene is excised and replaced with a therapeutic gene [3]. In comparison, gene replacement localizes the problematic gene and breaks it off using ZFNs or TALENs, creating a space for insertion of the healthy gene. Homologous recombination is used to perform the crucial replacement event. Homologous recombination occurs often throughout the chromosomes of a healthy body; it is the same mechanism used to repair broken or damaged DNA, or to introduce genetic variation from both parents during meiosis. Gene targeting therapies use homologous recombination to align the positions of the therapeutic gene with the patient's mutant gene and to replace them with one another.

Several selection processes are used to ensure that only successful products of this ex-vivo procedure are actually implanted. Whenever using homologous recombination in gene therapy, an antibiotic-resistance gene is added to the engineered construct region as a positive selection marker. In addition, a negative selection marker, the *tk* gene, is added to the periphery of the sequence's similarity region. The cells used for the transplant are grown in antibiotics to select for recombination, and in gancyclovir to kill any cells that carry random integrants. The antibiotic-resistance gene is then removed by a site-specific recombination system such as Cre/*Lox*P or Flp/*FRT* [4].

Arguably, this type of gene correction therapy is a safer and less cumbersome approach than gene addition; with gene correction, less genetic material is inserted overall. Moreover, the gene addition approach requires a mechanism by which the therapeutic gene can be expressed; an expression cassette containing a promoter must be inserted into the patient's genome, where it can activate the therapeutic gene. Gene correction only requires inserting the therapeutic gene and typically 34 additional base pairs.

Site-Directed Engineering of Replacement Genes in Stem Cells

Researchers have long been fascinated by the possibility of applying site-directed engineering to stem cells. Homologous recombination techniques prove most useful for these applications, since they allow for the targeted engineering of

replacement genes in the stem cells. In 1989, Schwartzberg et al. reported creating germline modifications in mouse stem cells, changes to the mouse's genome that would carry on to future generations, using homologous recombination-based genetic engineering [5]. In 1989, Mario Capecci won the Nobel Prize for a discovery making use of this method: the so-called "knockout mouse," valuable for researchers because of the opportunities it presents to study the effects of having a "knocked out" or absent version of a particular gene within an animal model. Knockout mice typically differ from wild-type mice by just one "knocked-out" gene; site-specific engineering makes such specificity possible.

The procedure for preparing knockout mice is a prime example of targeted gene therapy in action. First, embryonic stem cells are derived from non-genetically engineered mice. These stem cells are kept alive and cultured in a petri dish. The genetic area to be modified must be closely isolated and characterized during this period via the generation of subclones. An appropriate construct must be created, containing a selectable marker: for instance, antibiotic-resistance genes. When the cells are sufficiently cultured they are transfected with the construct. The cells must then be grown in a medium with antibiotics, allowing for the elimination of unsuccessful cells and the identification of successful ones. The successful cells express the desired gene.

These cells are then injected into blastocysts, embryos containing target clones, after which the blastocysts are transferred into pseudopregnant female mice (mice that are not pregnant but have hormonal levels that mimic the levels found in pregnancy). The result is the first generation of filial 0 (F0) chimeric mice that contain the modified genes. Chimeric mice are mated with unaltered mice, resulting in a filial 1 (F1) germline transmission of modified embryonic stem cells. These are the gene knockout mice that serve as the disease model [6, 7].

Of the species currently used in targeted gene therapy research, mice are the most similar to humans. Of late, homologous recombination methods have proven to be a powerful tool for elucidating gene function and have yielded insight into animal models of disease. The effects of lacking a particular gene on the phenotype of the mouse can help elucidate the effects of disease on human subjects. These can be used to test new treatments and drugs. For instance, in 1996, Chang et al. developed a mouse model featuring the inactivation of the alpha-globin gene using gene targeting to disrupt the 5′ alpha-globin gene of the mouse [8]. Since 1996, this model has been available for studies in hematology research. More recently, the p53 gene knockout mouse model has yielded information about perhaps the best known genetic lesion leading to human cancer, the mutation of the p53 tumor suppressor [9]. The use of knockout mice in ophthalmology research has become widespread.

While the mouse models created via homologous recombination and embryonic stem cells have proven extremely useful for drawing inferences about human diseases and treatment effects, there are still limits to the conclusiveness of mouse model studies. The cellular and physiological differences between mice and humans are too great to assume that genes are expressed similarly in mice and humans.

It is now becoming more feasible to create more targeted mouse models using homologous recombination to reengineer human embryonic stem cells (hESCs). Embryonic stem cell lines are self-renewable, with a high pluripotency that allows them to differentiate into almost all cell types [10]. Recently, homologous recombination techniques that use standard plasmid-based vectors to target endogenous genes have been tested on hESCs to see whether it is possible to modify them [11–17]. However, these procedures remain challenging in embryonic stem cells. To date, there have been few successful occurrences of homozygous disruption of a gene in hESCs. In addition, the natural rate of homologous recombination in genes is low, around one in 1×10^5 to 1×10^8 [18, 19], well below the standard for therapeutic use. It is necessary to raise the efficiency of the recombination event.

Song et al. have proposed one possible solution: the use of bacterial artificial chromosomes, vectors with large homologous arms that have been used to clone large sequences of DNA [20]. A recent study found that a BAC-based targeting vector was highly successful in disrupting targeted genes in hESCs.

Nonetheless, the use of artificial chromosomes poses a new challenge: it is difficult to confirm the homologous recombination event has taken place. Other recent studies have shown that the use of engineered enzymes to create a site-specific DNA double-strand break enables a high homologous recombination rate.

Zinc Finger Nucleases Increase Rates of Homologous Recombination in Gene Therapy

Zinc finger nucleases (ZFNs) are artificially engineered restriction enzymes created by fusing a zinc finger DNA-binding domain made up of Cy_2His_2 to a nonspecific DNA-cleavage domain, typically the type IIS endonuclease FokI [19]. The ZFNs' fusion proteins have been reported to stimulate localized mutagenesis and/or homologous recombination, while their DNA-binding domains provide high binding specificity to particular DNA sequences.

ZFNs contain two arrays, left and right, each consisting of three zinc fingers. Each zinc finger makes contact with three base pairs; each of the two arrays makes contact with about nine base pairs of the DNA sequence in total [19]. These fingers can be ordered in a multitude of combinations. Between the two arrays is a spacer region consisting of 5–7 base pairs, a cleavage domain derived from the type II restriction endonuclease FokI. This section of the zinc finger nuclease dimerizes and allows the ZFNs to bind to the target sequence. The FokI cleavage domain can recognize a target sequence of DNA of up to 23–25 base pairs in length and break each end of it, creating a double-strand break. These characteristics make ZFNs useful tools in homologous recombination.

The precise genome modifications created by ZFNs fall under two categories. A ZFN-induced double-strand break can result in non-homologous end-joining, causing small mutations such as insertions or deletions. Although these mutations

induced by ZFNs are not controlled, this technique is still used to create knockout cell lines in a variety of organisms. For example, in human disease research, non-homologous ZNF-induced breaks have already been used to add targeted mutations to human T cell *CCR5* genes, yielding T cells that are resistant to human immunodeficiency virus (HIV) infection [21]. This research has entered phase I clinical trials.

Secondly, ZFNs can modify the genome by creating site-specific DNA double-strand breaks and repairing these breaks via homologous recombination. In 2007, Lombardo et al. developed a technique for performing homologous recombination on hESCs using ZFNs [22]. Theoretically, this technique allows for making either small changes (on the order of a single nucleotide) or large ones (on the order of a large transgene cassette). Recent studies further demonstrated that the site-specific DNA double-strand break induced by ZFNs stimulates the homologous recombination process up to several 1,000-fold [23–27]. ZFN-induced homologous recombination, with its relatively high efficiency, is thus a promising tool for researchers, one with high therapeutic value for treating human disease [28–30].

However, even though ZFNs have demonstrated a high specificity when inducing double-strand breaks and have been shown to stimulate homologous recombination in vivo, their widespread adoption is still hindered by the lack of a robust, publicly available database for engineering zinc-finger arrays. The three zinc fingers of the ZFN can consist of a large variety of combinations that impact its binding specificity. A "modular assembly" approach could be used to join zinc finger modules into arrays. The procedures for accomplishing this are technically simple, but currently still inefficient, with a high rate of failure by trial and error. A formal database or library of ZFN, if made widely available, would present a solution to this problem.

In the past, ZFN creation methods have made use of oligomerized pool engineering (OPEN), an open-source, combinatorial sequencing-based protocol for building zinc fingers. OPEN utilizes the databases of the zinc finger pool, which determines the three base pair subsite of each zinc finger and randomly recombines them to form a random zing finger array library. However, the process of building and screening a combinatorial ZFN library is time consuming and labor intensive, limiting the broader adoption of this method [31].

The company Sangamo Bioscience has developed a platform for making four-zinc finger ZFNs. Although some detailed information about this method is already in publication, researchers must gain access to the proprietary database in order to be able to implement the ZFNs. The ZFNs developed from Sangamo Bioscience can be purchased from Sigma-Aldrich under the brand name of CompoZ. However, the limited accessibility of this source has limited its scope and scale.

A final method for generating ZFNs is provided by the context-dependent assembly (CoDA) platform. With this approach, two three-zinc finger arrays determined to contain a common middle zinc finger are assembled using N- and C- terminal fingers to form customized zinc finger arrays. The first finger is taken from one zinc finger array and the third finger is taken from the other. Nonetheless, this CoDA method constrains the identity of the middle zinc finger. It also leaves

unchecked the effects of the three zinc fingers on the affinity and the specificity of the final zinc finger array. For these reasons, this method is less preferable, especially in highly demanding therapeutic settings [32].

Overall ZFNs have been proven to have a high targeting specificity and many applications. However, the widespread use of ZFN-based gene therapy has been limited by multiple factors; namely, the high cost and the labor intensiveness of generating ZFNs.

Transcription Activator-Like Effector Nucleases (TALENs) Can Induce Site-Specific Double Strand Breaks

Induced double-strand breaks have a proven ability to disrupt gene sequences by two means: through non-homologous end-joining repair systems, or through homologous recombination repair pathways, with an exogenous plasmid as template. Like ZFNs, transcription activator-like effector nucleases, or TALENs, have been proven to induce double-strand breaks. TALENs are engineered DNA binding proteins which fuse transcription activator-like (TAL) effectors to a DNA binding domain. TALENs can recognize specific DNA sequences.

TAL effectors are produced by the *Xanthomonas* genus of plant pathogens. They activate transcription by binding to the effector sequence on the host cell promoter, once delivered into the host cells through type III secretion pathways [33]. The DNA binding domain of TAL effectors consists of tandem 33–35 amino acid repeat modules followed by truncated repeats of 20 amino acids. Among the amino acids in the DNA binding domain, the adjacent 12th and 13th amino acids are highly variable, while the remaining residues are nearly identical in each unit. These two high-variable amino acids, called repeat variable di-residues (RVD), are in charge of specifying the DNA binding target. Changing the RVDs makes the TAL effectors target different nucleotides with a high level of specificity.

Not only is the simple, straightforward sequence structuring convenient for predicting TAL effectors' DNA binding sites, but it also allows for the construction of custom TAL effectors [34, 35]. These engineered TAL effectors are proteins that fuse the TAL effectors with the catalytic domain of FokI nuclease: these are the transcription activator-like effector nucleases or TALENs. The FokI catalytic domain functions within a pair of dimers to create the double-strand break at the specific target sequence.

To generate customized TALENs to target specific sequences, previous research has suggested that the customized amino acid repeats of the DNA binding domain could be constructed through the sequential cloning of sequence-verified single, double, and triple repeat modules [36]. With this method, array integrity of TALENs' DNA binding domain repeats can be assured. Nonetheless, the proposed process is time consuming and labor intensive. Another alternative is to utilize methods based on a polymerase chain reaction (PCR) to generate sequences of

amino acid repeats in the DNA binding domain. This method would provide a faster and easier process for TALEN generation, but would also create a risk of sequence mutation or recombined repeats. Overall, the recently developed Golden Gate cloning technique has been reported to be the most efficient methodology for assembling multiple DNA fragment in an ordered fashion in one single reaction to generate customized TALENs [37].

Despite the relative newness of methods for using TALENs in gene targeting compared with the ZFNs, they provide many advantages. One study reports that the mutagenesis frequency of TALENs in transfected cells is estimated to be equivalent to or even 25 % higher than the rate in ZFNs [37]. Because of the simple structure of the TALENs, it is also easier to predict and manufacture customized TALENs to provide a high-targeting capacity. Moreover, TALENs have the ability to target some genes that have been reported as being particularly difficult to target with ZFNs.

Several studies have examined these unique properties of TALENs. In one study, Maeder and colleagues found dramatic differences in ZFNs' and TALENs' ability to locate and cleave to a three point deletion mutation associated with a cystic fibrosis transmembrane conductance regulator (CTFR). ZFNs were found to remain at least 120 base pairs away from the target site. Meanwhile, the target sequence resided well within TALENs' cleavage domain without compromising gene targeting efficiency [46]. Another study on targeting of the acetolactate synthase gene to create herbicide resistant tobacco plants found ZFNs at least 188 base pairs away from the desired side. The same study showed that TALENs cleaving to the site within 10 base pairs of the target sequence. Lastly, ZFNs have notable difficulties targeting the AT-rich regions in DNA. The same study showed that TALENs can successfully target two sites in the genome that contain 80.6 % AT [37].

These outstanding research results, combined with the TALENs' unique structural simplicity and manipulability make them not only an alternative of ZFNs for procedures inducing a site-specific DNA double-strand break, but also a remarkable tool for genome engineering on the whole. Nonetheless, issues such as TALENs' cleavage domain array length and need to customize high-affinity RVD arrays still remain to be addressed.

Viral Vectors Currently Used for Gene Delivery

Many different therapeutic gene delivering vectors have been developed since the beginning of gene therapy research in 1970s, including adenovirus vectors, lentivirus vectors, and herpes virus vectors.

Adenoviral Vector

Adenoviruses were first discovered in 1953. They are non-enveloped viruses; they consist of a protein core that contains a linear, double-strand DNA genome of

36–38 kb, and a large surrounding icosahedral protein shell, 70–90 nm in diameter. The protein shell is made up of 252 structures known as capsomeres: hexons, pentons, and fibers. The 12 pentons occupy the vertical part of the icosahedron. A slender projection called a fiber emerges from the base of each penton. The icosahedron has twenty faces made up of 240 capsomeres; these are called hexons because they form hexagonal arrays [38].

The adenovirus has several advantages as a delivery vector in gene therapy, including high transduction efficiency, high viral titer (10^{10} to 10^{13}), and a large insert size of up to 8 kb, due to its large genome structure. On the other hand, because the adenovirus does not have the capacity to integrate into the genome, any expression of the therapeutic gene with the adenovirus is not long-term, but transients. In addition, the adenovirus is a common human pathogen. Therefore, preexisting antibodies, or the induction of a human immune response, would hinder gene delivery via adenovirus vectors.

Lentiviral Vector

Research into the lentivirus started when human immunodeficiency virus type 1 was isolated in the 1980s. The lentivirus is a type of enveloped virus containing two copies of single-strand RNA in its viral core [39]. As research on the viruses has advanced, the development of lentiviral vectors has gone through three generations. The first generation of the lentiviral vector contains all the HIV-1 genes except for the envelope. This generation of lentiviral vector still contains HIV accessory genes in the packaging plasmid, so safety issues remain; the second generation of lentiviral vector removes the accessory genes of the HIV from the packaging plasmid, increasing the safety margin. Also, it retains the advantage of the first generation lentiviral vector it is able to infect both actively dividing and non-dividing cells.

The third, and current, generation of lentiviral vectors lacks the enhancer in the 3′ long terminal repeats (3′ LTR). This simpler genome consists of three genes: *gag*, *pol*, and *rev*. The gag gene codes for virion, the vector's main structural protein, while the *pol* codes for retrovirus specific polymerase/integrase and the *rev* is responsible for the post-transcription regulator, which ensures efficient *gag* and *pol* expression. The major drawback of the lentiviral vector is its potential to produce replication-competent viral vector, a dangerous event that is to be avoided. If used, this replication-competent viral vector would create insertional mutagenesis in the recipient of the gene therapy.

Herpesviral Vector

The herpesvirus vector is one of the relatively complex viral vectors used in gene therapy. The herpesvirus's central DNA core contains double-stranded DNA that

varies in size from 120 to 150 kb, with a molecule weight ranging from 80 to 150 million, depending on the specific virus. The DNA arrangement in the viral central core is still unknown. The DNA core is surrounded by an icosadeltahedral capsid consisting of 162 capsomeres; this stricture is around 100–110 nm in diameter [40]. The advantage of using the herpesvirus as a gene delivery tool is its high specificity and high transgenic capacity, which make it capable of carrying large therapeutic genes. Its high transgenic capacity provides an advantageous means for developing a variety of attenuated vectors. The most important ability of the herpesvirus vector lies in the way that it can develop nontoxic, latent and strong long-term therapeutic gene expression in neuron cells. However, the drawbacks of herpesvirus vectors are, firstly, that our immune system might develop immunity to herpesvirus infections, and secondly, a lack of packaging cells lines that makes it hard to construct herpesvirus vectors.

Adeno-Associated Viral Vectors

AAV vectors are utilized as a gene transferring tool due to their ability to produce targeted, long-term therapeutic gene expression. Also, the AAV vectors can infect both cells that divide quickly and slowly in vivo. However, the major defect of AAV vectors is their relatively small packaging amount, around 4.5 kb at the maximum. It is a rare event, but significant enough to note, that AAV vectors can integrate a small amount of viral gene during their integration, causing mutation in the host cells. Preexisting antibodies in the host immune system can hamper the efficiency of gene deliveries via AAV vectors.

Life Cycle and Genome Structure of Adeno-Associated Viruses

The AAV was first discovered in 1960s as a contaminant of adenovirus preparation. It belongs to human parvovirus family, and is one of the smallest viruses with a non-enveloped capsid, approximately 22 nm in size. The life cycle of AAV can be divided into two stages, the lytic and the latent, depending on the presence or absence of helper functions. In the lytic state, productive AAV is generated by either co-transfection of a helper virus or DNA-damaging agents. The helpers induce the change of the cellular environment to facilitate the AAV replication and gene expression. This results in the production of progeny viruses and the lysis of the host cell to release the newly produced AAV virions. In the latent state, in which helper is absent, AAV is not able to replicate and produce new virions. Instead, the AAV genome integrates into the host cell and is expressed by using host machinery. Once integrated into the host cell, the AAV genome can be activated by helper virus infection and start the lytic cycle to produce new virions [41].

There are many serotypes of AAV, the best studied of which is type 2 AAV (AAV2), which serves as prototype for the AAV family. Therefore, the general genomic feature of AAV will be discussed based on the AAV2 profile. AAV contains a linear single-strand DNA genome made of 4,680 nucleotides. The genome consists of two open reading frames, *rep* and *cap*, flanked by inverted terminal repeats (ITR) at the both ends of the DNA genome. The ITRs contain *cis*-element, which is required for replication, and can form a T-shaped, base-paired hairpin structure. Two genes, *rep* and *cap*, respectively, encode non-structural proteins for replication (Rep78, Rep68, Rep52, Rep40) and structural proteins for encapsidation (VP1, VP2, VP3). There are three promoters in the AAV, identified by their positions in the genome, p5, p19, and p40. And based on the AAV2 profile as general feature of the AAV family, all transcription has one single intron. Unspliced RNA encodes Rep78 and Rep52, while spliced RNA encodes the Rep68 and Rep40.

Recombinant Adeno-Associated Viral Vectors

So far, no human diseases have been found to be related to AAV, suggesting that wild-type AAV is nonpathogenic in humans. This safety is a great advantage for researchers who use AAV as a vehicle to introduce relevant genes as a therapeutic tool. As previously described, AAV is a helper-dependent viral vector.

To use AAV vector as a therapeutic tool, we must first produce a recombinant adeno-associated viral vector (rAAV) with helper functions. In wild-type AAV, only 5′ and 3′ ITRs are involved in the packaging process, which means that the entire wild-type AAV genome can be excised and replaced with a heterologous cassette consisting of a promoter, the therapeutic gene and a polyadenylation signal. To make rAAV, we need to triple transfect cells (for instance, 293 cells can be used) with three different plasmids. First, a single-strand AAV vector (ssAAV) is introduced, serving as a gene expression cassette flanked by ITRs at each end. Secondly, the AAV helper provides the capsid gene that targets specific receptors and the replication gene that triggers AAV replication. Third, an adenovirus helper plasmid provides *E2, E4*, and *VA* RNA genes. These three plasmids are transfected into cells that carry the adenovirus *E1* helper gene. After 48–72 h incubation, we can harvest rAAV from the transfected cells and purify with either iodoxanol gradient or CsCl gradient depending on the rAAV serotype. Finally, the purified rAAV is subjected to dialysis followed by characterization and then is ready to be used [42, 43].

Viral vectors are useful gene delivery vehicles for gene therapy; however, each of the viral vectors has its own pros and cons. Further research is needed to determine the best means to utilize the viral vectors without introducing side effects from the vectors themselves.

Future Perspectives on Gene Replacement Therapies

The process for inserting gene therapy into a patient or animal model directly, known as "gene addition therapy," was the first developed gene therapy technique that could treat inherited disorders.

Based on the foundations of the traditional gene therapy, gene correction therapy was later developed to "replace" the abnormal gene with the normal gene on the mouse model. Both of these gene manipulation techniques are not perfect and still must be improved. Safety issues involved in the use of viral vectors have limited treatments' applicability. Likewise, the use of homologous recombination technology for gene correction has been hindered by the low efficiency of recombination. Nonetheless, further research could hone a viral vector capable of safety and readily stimulating site-specific recombination in humans. The favorable biological features of recombinant AAV-derived vector and the discovery of induced homologous recombination technology lend high feasibility to these methods, which hold great value for many current research and future therapeutic applications.

The derivation of mouse embryonic stem (ES) cells combined with the gene targeting technology has helped develop our understanding of gene functions. Correcting genetic defects through homologous recombination is a technique would be useful for disease modeling and transplantation therapy in humans. Treatable diseases could range from diabetes and Parkinson's disease to even spinal cord injuries. Genes targeting would correct faulty genes in the cells, which could then be transplanted back into patients. The success of gene targeting in mouse ES cells has provided a means to evaluate gene functioning in vivo. In addition, faulty genes can be repaired by applying gene targeting technology to ES cells prior to transplantation therapies. Nevertheless, techniques in human cells must still be brought up to date with mouse-based technologies. Gene targeting techniques in human ES cells are still relatively immature.

The emergence of human-induced pluripotent cell technology has made gene manipulation into another platform for regenerative medicine. Induced pluripotent cells were first developed by successfully reprogramming mouse somatic cells with the four Yamanaka transcription factors: *Oct3/4, Sox2, Klf4*, and *Myc*. Using a similar strategy and the same transcription factors, human-induced pluripotent cells were successfully reprogrammed from adult human fibroblasts [39]. iPS cells are similar in morphology, proliferation, surface antigens, and gene expression to human embryonic stem cells. The high pluripotency of iPS cells makes them capable of differentiating into a variety of cell types in vitro. This characteristic of iPS cells not only makes them useful for understanding patient-specific disease mechanisms but for gene correction. In one prior study, Hanna et al. showed that iPS cells modified by homologous recombination were able to correct sickle cell anemia in a mouse model [44].

First, fibroblasts were harvested from a humanized sickle cell anemia mouse model. Cultured fibroblasts were transduced with the transcription factors *Oct4*, *Sox2*, *Klf4*, and *Myc* to induce them to become iPS cells. To correct the human

sickle β globin gene, a constructed template carrying wild-type β globin was introduced into the cell by electroporation, where it repaired the genetic defect through homologous recombination. The corrected iPS cells were then differentiated into embryoid bodies and further differentiated into hematopoietic progenitors in vivo. The corrected hematopoietic progenitors were transplanted back into the original donor mouse after irradiation, where they reversed the symptoms of sickle cell anemia [40]. The cured sickle cell anemia mouse model demonstrates that in mice, it is feasible to derive iPS cells, correct these with homologous recombination, and re-implant these into mice as a means of treatment. However, for the same advantages in mice to carry over to humans, the efficiency of homologous recombination must be improved; human iPS cells are extremely similar to human ES cells, in which rates of successful gene correction through homologous recombination have been shown to be low.

In the presence of an exogenously supplied donor template with sequence homology to the target region, homologous recombination is able to faithfully copy the donor templates into the endogenous loci, thus enabling incorporation of an exogenous sequence flanked by the two homology arms. Much research has focused on how to increase the efficiency of homologous recombination. By creating a DNA double-strand break at the specific locus using ZFN or TALEN technology, homologous recombination can be stimulated in vivo. In a study applying ZFN-based homologous recombination to primary cells from a mouse model with a generic recessive genetic disease, ZFNs were reported to create extremely precise genetic modification and also to stimulate the homologous recombination rate from 0.17 to 6.0 %, which could reach a maximal 1,000-fold stimulation over the targeting rate without ZFNs. Theoretically, this approach could be used to treat both dominant and recessive genetic diseases. The high specificity of ZFNs' targeting allowed controlled integration of the transgene and the direct correction of the disease-causing mutation. Only transient expression of ZFNs is required during the brief period of in vitro culture, while the genetic manipulation is present for the life of the cell, thus avoiding the need for continued expression of a foreign transgene.

In murine embryonic and adult fibroblasts, ZFNs have been shown to boost the rate of successful recombination to over 2 %. Thus the higher efficiency of ZFN-mediated homologous recombination offers an alternative method for correcting genetic defects in iPS cells. While typically, fibroblasts are induced to become iPS cells and then corrected, an alternative method would involve correcting fibroblasts directly with homologous recombination and then inducing fibroblasts to become stem cells. Future studies may be needed to compare the latter method with the former, which is currently the preferred one. Similarly, TALENs have been shown to efficiently induce DNA double strand breaks in vivo. Further study is needed to clarify how TALEN-mediated homologous recombination could be used to correct iPS cells.

Conclusion

Ex-vivo gene therapy involves generating stem cells from patients or animal models, correcting the disease-causing gene with gene therapy while cells are sustained in culture, and re-implanting the genetically altered cells into patients. Traditional gene therapy requires a viral vector to insert the therapeutic gene or genes into recipient cells. However, these viral vectors carry the risk of potential random genome integration, which could cause mutagenesis in the recipient cells. Site-directed homologous recombination technology provides an alternative platform for gene therapy. Gene correction therapy that used homologous recombination to directly replace disease causing genes, would minimize manipulation of the host genome. Nonetheless, the efficiency of the homologous recombination currently does not meet therapeutic standards, and would need to be improved.

ZFNs have been discovered to successfully elevate rates of homologous recombination by inducing a DNA double-strand break at a specific locus. The mutagenesis achieved through homologous recombination is thus stimulated by several 1 at the DNA double-strand break site. The widespread adoption of ZFNs is, however, hindered by the cost of manufacturing, largely due to lack of robust and publicly available database of zinc finger arrays. Recently, another type of enzyme was found to create a specific DNA double-strand break: the TALENs. TALENs are unique in their structural simplicity and manipulability and could be developed into an important tool for gene therapy.

Reprogramming adult fibroblasts into iPS cells has already been shown to be feasible; what remains is developing this technique into reliable and mainstream basis for ex-vivo gene correction therapy. However, the technical risks and challenges inherent in the methodologies involved must be overcome before iPS cells gain common application in therapeutic settings. The transcription factors used to reprogram adult fibroblasts into iPS cells should refined beyond the use of oncogenes, which might increase the risk of neoplastic transformation. In addition, the use of reprogramming retroviruses that increase the risk of insertional mutagenesis, should be closely monitored or avoided. Nonviral generation of iPS cells has become feasible [45]. The field continues to undergo rapid mutation and change; only the future will tell what direction gene therapy will take next.

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Chapter 11
Stem Cell-Based Therapeutics in Ophthalmology: Application Toward the Design of Clinical Trials

Rony Gelman and Stephen H. Tsang

Abstract Stem cell-based therapeutics have been proposed as a technology for restoration of anatomic structure and visual function for retinal degenerative diseases. Success in animal studies and preliminary trials may offer hope for patients afflicted by a variety of retinal degenerations. However, as clinical trials expand and advance to latter phases, it is important to address key study design issues. This chapter discusses the parameters for research into stem cell-based therapeutics. Efficacy endpoints for studies can be defined along objective physiologic, psychofunctional, anatomic, and functional living axes. Pupillometry, electroretinography, and radiologic tools are discussed as objective tools for the assessment of treatment outcome. Additionally, optical coherence tomography(OCT), fundus autofluorescence (FAF), and other imaging tools may be used. Psychofunctional tests may be less reliable among a pediatric population. Finally, improvements in functional living may be reported by patients and assessed by various measures.

Introduction

The normal human corneal epithelium is composed of flat stratified squamous epithelial cells. Goblet cells, which populate the conjunctival epithelium and are important as a source of mucin production for the tear film, are normally absent in the corneal epithelium. Normal corneal epithelium overlies a cuboid basal layer lying on the avascular corneal stroma. The population of epithelial cells that are

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S.H. Tsang (ed.), *Stem Cell Biology and Regenerative Medicine in Ophthalmology*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-5493-9_11,

located at the corneal limbus are commonly referred to as limbal stem cells and are responsible for the continued renewal of the cornea's epithelium [1, 2]. Various ocular pathologies that affect the limbal stem cells, such as chemical injuries, contact lens abuse, cicatricial pemphigoid, or Steven–Johnson syndrome, may lead to vision-threatening corneal compromise.

The human retina is a rich and complex neurosensory structure that depends on tight integration with other ocular structures for proper function. In particular, the neurosensory retina (composed of the retinal ganglion cells, the inner nuclear layer, and the photoreceptor layer) depends on healthy apposed retinal pigment epithelium (RPE) and interposed retinal vasculature. Although the retina does not have an intrinsic regenerative capacity, retinal stem cells are located in the RPE of the pars plana and pars plicata, and retinal progenitor cells are found in the ciliary margin zone. Diseases that compromise retinal vasculature, such as diabetes mellitus, may lead to loss of retinal neurosensory structures, and diseases that affect the RPE, such as age-related macular degeneration (AMD), may lead to secondary loss of photoreceptors.

Repopulation for unilateral or incomplete bilateral limbal stem cell deficiency has been achieved via autologous sources [3, 4]. Recent improvements in autologous limbal stem cell transplantation include the development of a temperature-sensitive culture dish and the use of amniotic membrane that may improve viability of the transplanted cells. In cases of bilateral limbal stem cell deficiency, various strategies have been utilized, including allogenic transfer combined with immunosuppression and transfer of cultured autologous cells from stratified epithelia of other areas of the body.

Targets for stem cell therapy in the retina include the vascular endothelial cells, the RPE, and the photoreceptors. RPE cell replacement has been studied in clinical trials, mostly with limited results [5, 6]. The generation of a spontaneous immortalized RPE cell line and successful retinal cell transplantation into rodent models of retinal degeneration offer promise for retinal repair.

Stem Cell-Based Therapeutics

Stem cell-based therapeutics have been proposed as a technology for restoration of anatomic structure and visual function for retinal degenerative diseases. Success in animal studies and preliminary trials may offer hope for patients afflicted by a variety of retinal degenerations. However, as the clinical trials expand and advance to latter phases, it is important to address key study design issues that will be explored below.

The following material was adapted from the Federal Drug Administration (FDA) Cellular, Tissue and Gene Therapies Advisory Meeting held on June 29, 2011 [7], in particular the discussion led by Dr. J. Timothy Stout (Casey Eye Institute) [also Stout and Francis [8]]. Key questions were posed regarding study design, in particular: (1) the definitions of efficacy endpoints; (2) safety concerns; and (3) drug administration. In the following sections, we will review each of these three components.

Efficacy Endpoints

Efficacy endpoints can be defined along objective physiologic, psychofunctional, anatomic, and functional living axes.

Objective physiologic endpoints are not dependent on patient feedback, and thus are not subjective. One such test is pupillometry, for which there are available commercial testing units that are well validated, sensitive, and reliable; they are a good modality for use in both adult and pediatric patients. Pupillometry can be helpful for diseases affecting the entire retina, but may be difficult to use in cases of nystagmus. Another objective physiologic endpoint can be measured by nystagmography, which is similar to pupillometry, is commercially available, sensitive and reliable, and provides a good modality for both adults and children. However, most ophthalmologists do not routinely use this test, and its use would likely be limited to those severe disease cases associated with nystagmus.

Electrophysiology is another available objective physiologic modality. The electroretinogram (ERG), visually evoked potential (VEP), and multifocal ERG (mfERG) have been implemented by commercial systems for adult and pediatric patients, but complications may include imperfect standardization between centers as well as issues of reliability and validity. Moreover, these techniques have not been commonly used for endpoint analysis. Below, we discuss the features of this type of imaging in evaluating the outcome of treatment.

Alternatively, radiologic tools such as functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) may be useful technologies to incorporate, but their availability may be limited to certain centers and their use in the pediatric population may be constrained. In addition, their use for ophthalmic protocols has not been validated and therefore will need further study.

Unlike physiologic endpoints, psychofunctional endpoints require patient feedback for the interpretation of results. Visual acuity is the most fundamental measurement. Although often considered a "gold standard," visual acuity testing may be a less reliable measure in the pediatric population.

Color vision testing is readily commercially available, but the validity and reliability as a measure of disease progression or therapeutic response remain to be studied. As with visual acuity testing, reliability will be reduced when testing children. In addition, a small percentage of the population may have preexisting color vision deficits, thus potentially excluding this test from that patient subset.

Contrast sensitivity is another psychofunctional technique that is readily available and has previously been validated for optic nerve disease trials. Its use, however, may be limited in the pediatric population and in adults that are affected by ocular media issues such as cataract.

Lastly, visual field testing is a commonly used psychofunctional test that is implemented by many commercial systems. It has been well validated and can discriminate between central versus peripheral disease-specific patterns. However, its utility is limited by poor reliability with patient fatigue or in patients too young to accurately complete the test.

Anatomic endpoints are largely based on well-established and recently developed imaging techniques. Digital fundus photography and fluorescence angiography are widely available and validated, although their use may be limited in cases of nystagmus or in the pediatric population.

Optical coherence tomography (OCT) has become a more widely used and validated imaging modality. However, some centers may not have access to the newest high-resolution and high-speed spectral domain systems. In addition, patient cooperation difficulties due to age or nystagmus may limit the utility of OCT.

Fundus autofluorescence (FAF) is becoming a more commercial imaging technique that can potentially provide quantitative characterization of autofluorescence patterns in retinal diseases. Although some commercial spectral domain OCT systems have integrated FAF imaging, some centers may lack access to this imaging modality. Moreover, its utility may be limited in cases of nystagmus or young children.

Adaptive optics (AO) offers a powerful technique to measure retinal morphology, including cone density and spacing, and to potentially discern differences in various retinal diseases or to measure responses to therapy. Its limitations, however, are that there are few commercially available systems available and that poor fixation in cases of nystagmus or poor patient cooperation severely limit its utility.

Functional living endpoints include patient reported outcomes (PROs), mobility testing, and reading performance metrics. PROs, such as the VFQ51, VFW25, and Visual Function Index, are validated but subject to patient bias and would have limited use in the pediatric group. Mobility testing is not standardized or commonly used, but may be a useful test for monitoring outcomes. Reading performance metrics are validated tests that are commercially available but may be affected by patient fatigue. This test is probably best suited for adults affected by macular disease.

The ERG in Electrodiagnostic Imaging

Electroretinography is a tool for measuring the electrical impulses of neurons. Photoreceptors and downstream neurons in the retina maintain a non-neutral electrical "resting potential" by manipulating the intracellular and extracellular concentrations of positive sodium, potassium, and calcium ions and negative chloride ions, as well as larger electronegative molecules.

Human rod cells present a model system of phototransduction. The chromophore, or light-sensing pigment, in rods is 11-*cis*-retinal, which is bound to an apoprotein called opsin, forming rhodopsin. When a photon strikes 11-*cis*-retinal, the added energy causes it to isomerize into all-trans-retinal [10, 11, 12, 13]. This conformational change causes rhodopsin to activate transducin, a heterotrimeric G protein [14, 15, 12]. Activated transducin binds to the inhibitory subunits of phosphodiesterase 6 (PDE6), thereby de-inhibiting it. The newly active PDE6 hydrolyzes cyclic guanosine monophosphate (cGMP), reducing intracellular cGMP levels and closing cGMP-gated cationic channels (CNG) in the rod cellular

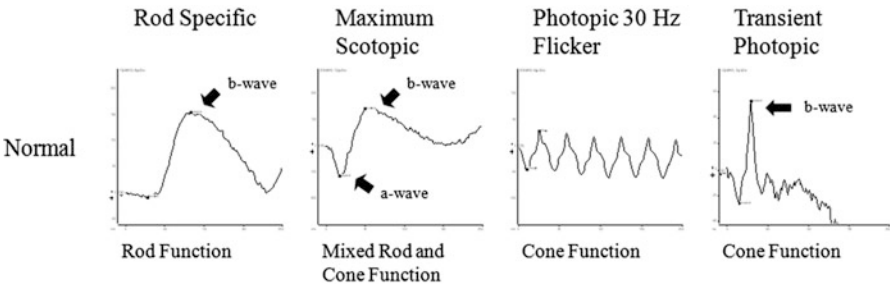

Fig. 11.1 Normal electroretinogram (ERG) Tracings. The a-wave and b-wave are noted where applicable

membrane [16, 11, 17, 13]. This reduces the influx of Na^+ and Ca^{2+} into the cell, thereby hyperpolarizing it.

The hyperpolarization of the cell causes it to cease transmitting glutamate across synapses to bipolar cells, inducing changes in their polarization. Bipolar cells transmit this signal either directly to ganglion cells, each of which has an axon proceeding out of the orbit along the optic nerve, or to amacrine cells, which then activate ganglion cells or alter the output of other bipolar cells. Photoreceptors, bipolar cells, and amacrine cells operate via graded potentials, but ganglion cells generate action potentials in response to incoming signals from bipolar and amacrine cells; these action potentials help to propagate the information along the optic nerve. The function of each of these cell types can be measured using precise electroretinographic techniques.

Wave Components Explanation

The typical ERG waveform (see Fig. 11.1, Maximum Scotopic) is the sum result of activity in the photoreceptors and bipolar cells, with some contribution from Müller cells. The initial negative deflection, known as the a-wave, is the result of early signals from the rod and cone photoreceptors. The subsequent rise toward the positive peak, known as the b-wave, is created primarily by slower signals from the rod and cone bipolar cells. The ascending slope from the a-wave to the peak of the b-wave typically shows several small oscillations; these are called the oscillatory potentials, or OPs, and reveal the function of the amacrine cells. Other components that become apparent only under certain conditions are beyond the scope of this chapter.

Safety Concerns

The normal human eye is generally considered an immune-privileged organ, but one of the concerns associated with the intraocular administration of a potential gene or stem cell-based therapy is the development of an immune-mediated response after repeat or contralateral eye dosing. Preclinical studies in animals

may help to predict the immune response, but may be problematic because response may vary with animal species, specific therapy administered, site of administration, injection technique, host immune response, timing of the contralateral dose, use of immunosuppressive agents, and intra-subject eye disease.

The risks associated with repeat or contralateral dosing can be possibly minimized. Suggested strategies include general safety and adverse reaction surveillance, specific monitoring for an immune response, staggering patient enrollment, adjusted administration intervals, and immunosuppressive regimens targeted toward reducing risk, although none of the suggested strategies have been well established or validated.

Preliminary investigation of readministration of recombinant adeno-associated virus (AAV) carrying the *RPE65* gene in three patients with Leber congenital amaurosis 1.7–3.3 years after they had received their initial subretinal injection indicate that readministration is both safe and efficacious after previous exposure to the vector [9]. Further work is warranted to characterize the safety and efficacy of readministration of gene products.

Drug Administration

Many varied delivery methods for gene therapy or stem cell-based therapeutics exist. In this section, we will review the following methods: systemic, topical, transscleral, anterior chamber, intravitreal, subretinal, and suprachoroidal.

Systemic delivery has the advantage of being minimally invasive; however, it has an multiplicity of infection. some viral inactivation may occur, and safety concerns such as promoter control and widespread integration may exist. Systemic delivery is inappropriate for cell-based therapies.

Topical delivery is minimally invasive, but also minimally effective due to the very low levels of transduction through conjunctival and corneal epithelia and the lack of transduction to the posterior pole. The use of collagen shield may aid enhancement. Topical delivery is inappropriate for cell-based therapies.

Transsceral and transcorneal deliveries are minimally invasive as well, but suffer from low transduction efficiency. Iontophoresis is an established methodology in drug delivery that is likely to be less effective for viral vectors. Transsceral and transcorneal deliveries are inappropriate for cell-based therapies.

Delivery via the anterior chamber is minimally invasive with some transduction effect on the trabecular meshwork endothelium, corneal endothelium, ciliary body endothelium, and iris epithelium. No transduction to the posterior segment is achieved by this delivery method, rendering it inappropriate for cell-based therapies.

Intravitreal delivery is minimally invasive and has become a standard of care in the vast majority of retinal practices with the introduction of anti-VEGF intravitreal injections. Good transduction of the ciliary body epithelium is achieved. Stem cells introduced by this delivery method may proliferate, possibly leading to epiretinal membrane formation.

Standard subretinal delivery is an invasive procedure, requiring a vitrectomy with a posterior retinotomy. Excellent transduction of photoreceptors and RPE can be achieved, and this methodology is the current standard of care for gene delivery. Disadvantages of this delivery method include unpredictable bleb development and unpredictable efflux of product with a posterior retinotomy, as well as possibly higher complication rates.

Subretinal delivery can also be accomplished via an ab externo entrance. No vitrectomy or retinotomy is required. This method has a steeper surgical learning curve compared to the standard subretinal delivery method and is not validated at this time.

The suprachoroidal delivery method is minimally invasive and conceivably can be performed as a procedure in the clinic. Excellent transduction of the choriocapillaris and choroid can be achieved. Though this method is appropriate for gene and cell-based therapies, its validity remains to be investigated further.

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S.H. Tsang (ed.), *Stem Cell Biology and Regenerative Medicine in Ophthalmology*,
Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-5493-9,

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S.H. Tsang (ed.), *Stem Cell Biology and Regenerative Medicine in Ophthalmology*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-5493-9,