Stem Cell Biology and Regenerative Medicine

Alice Pébay *Editor*

Regenerative Biology of the Eye

Humana Press

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ISSN 2196-8985 ISSN 2196-8993 (electronic)
ISBN 978-1-4939-0786-1 ISBN 978-1-4939-0787-8 (eBook)
DOI 10.1007/978-1-4939-0787-8
Springer New York Heidelberg Dordrecht London

Library of Congress Control Number: 2014939607

Printed on acid-free paper

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

Acknowledgments

This volume in the *Stem Cell Biology* and *Regenerative Medicine* series presents the current knowledge on various aspects of the potential regenerative biology of the eye.

I would like to thank all the authors of this volume who have shared their expertise. I also wish to thank Dr. Kursad Turksen for his support during the process of compiling this book. Finally, a special thanks goes to Aleta Kalkstein for her help during the preparation of the volume.

Melbourne, Australia — Alice Pébay

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Chapter 1
Understanding Retinal Development Can Inform Future Regenerative Therapies

Peter D. Westenskow

Contents

Abbreviations

AMD	Age-related macular degeneration
bHLH	Basic helix-loop-helix
bHLH-Zip	Basic helix-loop-helix leucine-zipper protein
CA	Constitutively-active
CNS	Central nervous system
DN	Dominant-negative
ES	Embryonic stem cell
FGF	Fibroblast growth factor
HIF	Hypoxia-inducible transcription factor
iPS	Induced pluripotent stem cell

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A. Pébay (ed.), *Regenerative Biology of the Eye*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4939-0787-8_1,

MacTel	Macular telangiectasia
MAPK	Raf-MEK-mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
Mitf	Microphthalmia-associated transcription factor
OC	Optic cup
orJ	Ocular retardation mouse
Otx2	Orthodenticle homolog 2
OV	Optic vesicle
Pax	Paired box gene
PLE	Presumptive lens ectoderm
RAP	Retinal angiomatous proliferation
RGC	Retinal ganglion cell
RPC	Retinal progenitor cell
RPE	Retinal pigment epithelium
SE	Surface ectoderm
Shh	Sonic hedgehog
TGF	Transforming growth factor
Tyrp2	Tyrosinase-related protein 2
VEGF	Vascular endothelial growth factor
VHL	Von Hippel Lindau
Vsx2	Visual system homeobox 2

1.1 Introduction

The first step for generating vision, phototransduction, occurs in the sensory retina where light energy is absorbed, converted into electrical impulses, and transmitted to the brain. Phototransduction occurs in the specialized outer segments of rod and cone photoreceptors where the light-responsive vitamin-A-derived chromophores (11-*cis*-retinal) are housed in opsin proteins. When 11-*cis*-retinal absorbs a photon, it is isomerized to all-*trans*-retinal; this activates a cascade that results in hyperpolarization of the cell. Interneurons in the inner cellular layer refine the outputs and send them to the ganglion cells, whose axons bundle and transmit the integrated signal to the visual cortex. To reinitiate the process retinal isomers in the all-*trans* state must be reisomerized by neighboring retinal pigment epithelium (RPE) or Mueller glia cells. Deficits in this process can induce retinitis pigmentosa, a group of genetic eye conditions that result in primary photoreceptor cell death and incurable blindness (for review, see [1]).

RPE cells provide essential trophic and functional support to the retina and vasculature (for review see [2]). They control various aspects of eye development including dictating the rate of eye growth, formation of the ocular circulatory system, regulating neurogenesis and synaptogenesis, and iris development [3–7]. The diverse functional capacities of the RPE are remarkable, and they are ideally positioned between the photoreceptors and circulation to regulate several key

functions indispensable for vision. RPE cells are highly polarized and extend long microvilli from their apical surfaces towards the neural retina that wrap around the photosensitive tips of the rod and cone photoreceptors. They generate the outer blood retinal border, regulate adhesion, osmolarity, pH, and water balance in the subretinal space. RPE cells absorb light to prevent scatter, are largely responsible for maintaining the relative immune privilege of the eye, and diurnally phagocytose light-damaged membranes and proteins in shed photoreceptor outer segments.

RPE and photoreceptors are so codependent that they are considered to be one functional unit. RPE cell dysfunction or death invariably induces secondary photoreceptor degeneration. In fact RPE cell dysfunction or atrophy is characteristic of age-related macular degeneration (AMD). AMD is the leading cause of blindness in industrialized countries [8, 9], and demographic analyses predict that it will become even more widespread in upcoming years [10].

Additionally, glial cells are required for maintaining retinal homeostasis, and iris and ciliary body cells regulate light exposure and ocular pressure. Glia are a diverse group of CNS-specific connective tissue cells that exist as macroglia, Mueller glial cells) and microglia. Primate retinas are highly vascular and, besides the choriocapillaris, that supplies blood to the outer third of the retina, have three distinct intraretinal plexus layers to support the inner retinal neural networks.

The more we understand about how retinal cells develop and function the better equipped we will be to design sound therapeutic interventions for the diseases listed above. There are several specific reasons why researchers specialized in ocular translational medicine should have a strong background in eye development. (1) To understand the intrinsic networks and molecular bases of retinal diseases. This knowledge could help design more effective and creative gene therapies. (2) Researchers must be able to rigorously characterize stem cell-derived cells compared with their primary counterparts to determine if they exhibit sufficient structural and functional similarities. (3) Delivering cocktails of signaling factors to differentiating stem cells in chronological sequences in to recapitulate developmental pathways in vitro *may* improve yields and result in the safest end products. (4) A thorough understanding of the retinal cell's microenvironments, how the microenvironments are established and affected by aging and/or disease, and which neighboring cells they interact with during development, all could provide researchers with critical clues for optimizing cell replacement-based therapeutics.

The focus of this chapter is to provide a basic review of retina development and to provide a perspective on how this will inform future therapies. A strong emphasis will be placed on RPE and photoreceptor development, including step-wise development models, since cell replacement endeavors (especially for RPE cells) are rapidly moving towards the clinic (for review see [11]). The derivation of photoreceptors from stem cells and successful implantation, while riddled with complex technical challenges, represent perhaps the best hope for a cell-based approach for *restoring* vision loss. Since the biggest challenge facing photoreceptor transplantation may be dealing with the aftermath of retinal remodeling that occurs when photoreceptors become stressed, a brief primer on retinal remodeling will be included. Development of Bruch's membrane and the retinal and extraretinal vasculature will

also be briefly outlined since cell transplantation strategies for both cell types are likely to be ineffective unless proper microenvironments either exist or can be reestablished. The chapter will conclude with a discussion about how common themes and observations made during development can help guide the design of effective tissue-engineering and transplantation approaches.

1.1.1 Basic Overview of Retina Development

Eye development proceeds in a well-coordinated series of morphogenic movements and dynamic changes in gene expression that are guided by intrinsic and extrinsic cues [12–14]. Progenitor cells are specified to generate the primordial eye field in the anterior neural plate after gastrulation and expand bilaterally in a neuroectoderm layer to form the optic vesicles. Neighboring mesenchymal cells secrete various signaling molecules to pattern the vesicle into presumptive RPE (dorso-proximal region), and neural retina (distal region) by activating genes important for RPE development (including *Mitf*/*Otx2*) or neural retina (*Vsx2*). Other key patterning molecules include Activin-like signals that activate the *Mitf* gene to induce RPE [15]. Fibroblast growth factor (FGF) signals, perhaps secreted from the lens ectoderm, repress *Mitf* and may induce *Vsx2*. Vsx2 inhibits *Mitf* and is the earliest known marker of retinal progenitor cells (RPCs) [16–18]. Shh is also important for specifying the ventral RPE, perhaps by regulating *Otx2* expression, and for ventral patterning of the retina [19, 20]. Later, Wnt/β-catenin signaling is required for maintenance of *Mitf*/*Otx2* expression and RPE fate [21–23].

Other refinements to gene expression profiles are also critical for eye development. *SoxB1* genes, which are important for regulating neural retina competence, are expressed broadly in the optic vesicle but become restricted to the neural retina [24]. *Pax2* and *Pax6* genes, which are required for optic stalk development and neural retina development, are initially expressed in the entire vesicle but become restricted to the optic stalk and neural retina, respectively [25, 26]. Additionally, *Pax2* and *Pax6* reciprocally inhibit each other's expression, and Shh delivered from the ventral midline upregulates *Pax2* in the optic stalk and inhibits *Pax6* to sharpen the neural retina and optic stalk boundary [26, 27].

It is important to note that both neural retina and RPE cells are bipotent through early development in species-specific windows and can be "respecified" to either RPE or neural retina cells [17, 18, 28, 29]. Consequently, maintenance of cell-fate is critical and fate decisions must be continually reinforced during development [3, 30]. Maintenance of RPE fate will be addressed in a later section.

An invagination of the distal part of the vesicle creates a two-layered structure called the optic cup. The neural retina will be generated from cells in the inner layer and RPE cells will form from the outer layer. Some of the molecular pathways that guide development of both structures will be outlined individually in the next sections.

The RPE develops from the outer layer of the optic cup. As they continue to differentiate, RPE cells increase their cell surface area by generating actin-rich

apical architecture and long mature microvilli that serve to direct the elongation of the photoreceptor outer segments and by generating basal infoldings [31–33]. Final maturation occurs based on their location in relation to the retina to adapt to specific functional requirements of photoreceptors at that position. This is especially apparent over the macula where RPE cells are smaller (roughly 20 % in diameter), synthesize more melanin, and organize their melanosomes differently than cells outside the macula [2].

Neurons and glia are generated in a highly conserved birth order from RPCs in the inner layer of the optic cup (for review see [34]). This occurs as the RPCs respond to environmental cues that alter their gene expression profiles at discrete developmental time points. The result of the activation of complicated gene networks in discrete subpopulations of progenitor cells is the generation of six retinal neurons and Mueller glia in stratified cellular layers (for review see [35]). Terminal differentiation of photoreceptors occurs once opsin expression is potentiated, outer segments form, and synaptic connections are made with retinal interneurons.

1.1.1.1 Intrinsic Regulators of Retina Development

Very little is known about how the retina develops and what factors are important for specification, morphogenesis, and especially maintenance of cell-fate. A few key intrinsic factors have been shown to positively regulate RPE development (*Mitf*, *Otx2*, *Pax* genes, and *Gas1*) and others have been shown to promote neural retinal cell development and suppress regulate RPE fate (*Vsx2* and *SoxB1*). The effects are briefly summarized in Table 1.1.

Microphthalmia-Associated Transcription Factor (Mitf)

Mitf is a basic helix-loop-helix leucine-zipper protein (bHLH-Zip) that is required for the development of neural crest-derived melanocytes and RPE, and binds to E-box sites to transactivate many pigment synthesis genes and genes important for building mature melanosomes including *Tyrosinase*, *Tyrosinase-related protein 2* (*Tyrp-2*), *dopachrome tautomerase* (*Dct*), and the melanosome glycoprotein *QNR71* [36]. Mitf also regulates *Bestrophin* (*VMD2*) that encodes an important chloride channel in the RPE [37].

Mitf mutations in humans can result in Waardenburg Syndrome Type II and Tietz Syndrome that are marked by deafness and generalized hypopigmentation, and *Mitf* is shown to regulate all but one of the identified genes that are linked to albinism [38–44]. Mutations in mouse and avian gene products display gross RPE pigment defects: the RPE cells begin hyperproliferating, and retinal markers are upregulated in RPE domains in a process termed RPE-to-retina transdifferentiation. *Mitf* gain-of-function experiments in cultured quail retinal cells induce pigment synthesis, and in the chicken neural retina induce ectopic pigmentation and activation of downstream Mitf target genes [45–47].

Table 1.1 Intrinsic regulators of retina development

Factor	Expression pattern	Function(s)	Experimental perturbations
Otx2	Pan-vesicular in OV, downregulated in presumptive neural retina of late OV stages [50, 66]	Activates RPE-specific gene expression. Important for OC morphogenesis [45, 49, 50]. Required for photoreceptor development [54, 146]	RPE-to-retina transdifferentiation and ventral eye overgrowth induced when one allele is mutated in an *Otx1* null background. Lens, optic nerve, and OV patterning are also affected [50]
Mitf	In mouse, pan-vesicular in OV, in chick only induced in presumptive RPE of late OV stages [18, 46]	Activates RPE-specific gene expression including many pigment synthesis genes [36, 37]	RPE-to-retina transdifferentiation and dorsal overgrowth in mutants; failure of the optic fissure to close, optic nerve defects are also observed [197, 31]
Pax6	Pan-vesicular in OV, transiently expressed in RPE [25]	Along with Pax2 activates *Mitf-A*, regulates the rate/onset of RPE differentiation [25, 72, 73]. Can be pro-RPE or pro-retina depending on available co-factors [48]	No RPE phenotype observed unless combined with *Pax2−/−* alleles, then RPE-to-retina transdifferentiation occurs. Pax6 overexpression in Pax2 domains induces optic stalk-to-RPE transdifferentiation [25]. Pax6 overexpression prevent RPE to retina transdifferentiation in *Mitf* mutants [48]
Pax2	Pan-vesicular in OV, restricted to OS in OC stages [25]	Responsible for optic stalk development; activates *Mitf-A* [25]	No RPE phenotype observed unless combined with *Pax6−/−* alleles, then RPE-to-retina transdifferentiation is observed [25]
Gas1	Expressed in ventral OC [198]	Unknown	RPE-to-retina transdifferentiation observed in only ventral RPE domains of mutants [198]
Vsx2	Induced in presumptive neural retina in later OV stages, and in bipolar cells [18, 75]	Maintenance of retinal progenitor cells states, represses *Mitf-D* and *-H*, OV patterning [16, 17, 76]	Microphthalmia in mutants accompanied with ectopic Mitf expression and pigmentation in peripheral domains. Misexpression in RPE induces pigment defects [17, 76]
SoxB1	Pan-vesicular, downregulated in presumptive RPE [24]	Regulates neuronal competence [199]	Prolonged or forced expression inhibits RPE differentiation. Forced *DN-SoxB1* expression induces *Mitf* expression in chicken neural retina [24, 200]. The conditional depletion of *Sox2* in the mouse retina does not induce Mitf or Otx2 [199]

OV optic vesicle, *OC* optic cup, *OS* optic stalk, *DN* dominant-negative

The *Mitf* gene does not encode a single gene-product, but a family of isoforms generated through alternative promoter and exon use. *Mitf-A*, *-J*, *-H*, and *-D* are expressed in the RPE but *Mitf-D* and *-H* are probably the most important [16] although the RPE of adult transgenic mice lacking *Mitf-D* appear completely normal. However, the expression of the other *Mitf* isoforms (especially *-H*) is compensatorily upregulated and may substitute for the loss of *Mitf-D* [48].

The expression of Mitf must be carefully maintained during development to maintain RPE fate. As one example, the implantation of FGF-soaked beads, that repress RPE fate, near the optic cup in chicken induces RPE-to-retina transdifferentiation [28]. If *Mitf* expression is ectopically enhanced either in vitro or in vivo, FGF-induced RPE-to-retina transdifferentiation may be prevented thereby demonstrating that maintenance of *Mitf* expression is imperative for RPE development [29, 46]. We have shown that co-transfection of neural cells in vivo with *β-catenin* and *Otx2* can activate ectopic Mitf expression [23]. Additionally, *Mitf-D* and *-H* are directly regulated by the Wnt/β-catenin signaling pathway [21, 22]. These observations suggest that the Wnt/β-catenin pathway is a potent regulator of *Mitf* and of RPE fate.

Orthodenticle Homolog 2 (Otx2)

The *Otx2* gene encodes a member of the bicoid subfamily of homeodomain-containing transcription factors. Similar to *Mitf*, *Otx2* can transactivate the *Tyrosinase*, *Dct*, *QNR71*, and *VMD2* genes [45, 49]. Otx2 may act as a competence factor that allows RPE specification when combined with pro-RPE factors such as the Wnt/β-catenin pathway. As mentioned above, combinations of *Otx2* and *β-catenin* transgenes in chicken neural retina cells activate *Mitf* in vivo. This synergism occurs in an additive manner based on in vitro analyses [23]. Based on these observations and several others, not only is *Otx2* required for RPE development, but that the dosage of *Otx2* may be important. Otx2 deficits can induce pronounced pigment deficits, RPE-to-retina transdifferentiation, optic vesicle patterning, and optic cup morphogenesis defects [50]. Mitf is no longer detectable in the mutant RPE (neither is Otx2 observed in the RPE of *Mitf* mutant mice) suggesting that Mitf and Otx2 may regulate each other's expression [50]. *Otx2* transfection in quail dissociated retinal cells induces pigmentation and in some cases, *Mitf* expression [45]. In humans *OTX2* haploinsufficiency has been implicated in multiple eye disorders including cases of microphthalmia and anophthalmia [51].

Otx2 is initially expressed broadly in the optic vesicle before becoming restricted to the presumptive RPE prior to invagination. Its expression is maintained in the RPE through adulthood, and is reactivated in the neural retina in developing photoreceptors and bipolar cells [52–56]. It is also detected in interneuron and relay cells in the retina.

As its broad and dynamic expression pattern implies, *Otx2* is required for many diverse capacities. It is required for eye-field specification, photoreceptor differentiation (described in more detail later), opsin gene regulation, and for regulating many of the genes of phototransduction gene networks [45, 49, 57–60].

Overexpression of Otx2 in the neural retina of mouse or in stem cells derived from rat iris and ciliary body of rat results in overproduction of photoreceptors [52, 54]. In frog, however, Otx2 overexpression results in an overproduction of bipolar cells, implying that it may have different roles in different organisms [61].

The *Otx2* gene product is also a family of isoforms generated through alternative promoter and exon use [62]. To date, in mouse four isoforms have been identified that differ only in their N-terminal noncoding sequences [62–64]. While *Otx2-D* has not been well characterized yet, *Otx2-A*, *-B*, and *-C* are all expressed in the mouse RPE and neural retina in adult stages [64]. Despite differing only noncoding exon sequences, selective inactivation of all four *Otx2* isoforms reveal in their specific requirements for visceral endoderm anteriorization [62]. Whether *Otx2* isoforms have RPE-specific functions remains to be determined. Additionally, three enhancers have been characterized: *Otx2T1* that is upstream of *Otx2-C*, *Otx2T0* that lies upstream of *Otx2-A*, and *Otx2 FM2*. We have shown that the *Otx2T0* enhancer can be activated by mouse RPE cells in vivo [23]. The remote *Otx2 FM2* enhancer drives *Otx2* expression in the mouse RPE, midbrain, and forebrain [65].

Despite its prominent functions in the RPE, it is unclear how *Otx2* is regulated, especially how its expression is maintained in the presumptive RPE but repressed in the presumptive neural retina during late optic vesicle stages [50, 55, 66]. Another unresolved question is how Otx2, which has broad abilities to specify many different and very diverse cell-type differentiation programs, can operate in the RPE to activate only RPE differentiation genes. One theory is that *Otx2* may integrate its transcriptional abilities with *Mitf*. In fact, Otx2 and Mitf have been demonstrated to physically interact and co-expression of both factors results in synergistic activation of RPE-specific gene promoters [50]. Consequently, they may function more effectively in combination, perhaps explaining why either *Mitf* or *Otx2* mutants induce dramatic RPE deficits even though the other factor is, at least initially, present. Additionally, Otx2 may be required to mediate the effects of Wnt/β-catenin signaling to induce RPE-specific gene expression by perhaps "priming" the optic vesicle and establishing RPE competence. Competence may be established, for example, by activating genes for signaling pathway receptors or for other transcription factors that it may operate through. Or it may be established by relieving epigenetic repression so that other transcription factors may bind and activate their target genes [67].

In other systems Otx2 drives specific cell-fate decisions during embryogenesis by operating with extrinsic and intrinsic co-factors [68]. In the eye Otx2 integrates with Notch/Delta signaling and NeuroD transcription factors to regulate lens and photoreceptor development respectively (see Fig. 1.1) [69, 70]. Collectively, these data imply that the effects of Otx2 in the RPE and retina are likely mediated by different co-factors.

Paired Box Genes (Pax2 and Pax6)

Pax genes are transcription factors that contain both paired domains and homeodomains, and are important for the specification of many tissues both in and outside of the developing nervous system (for review see [71]). Dramatic RPE deficits are

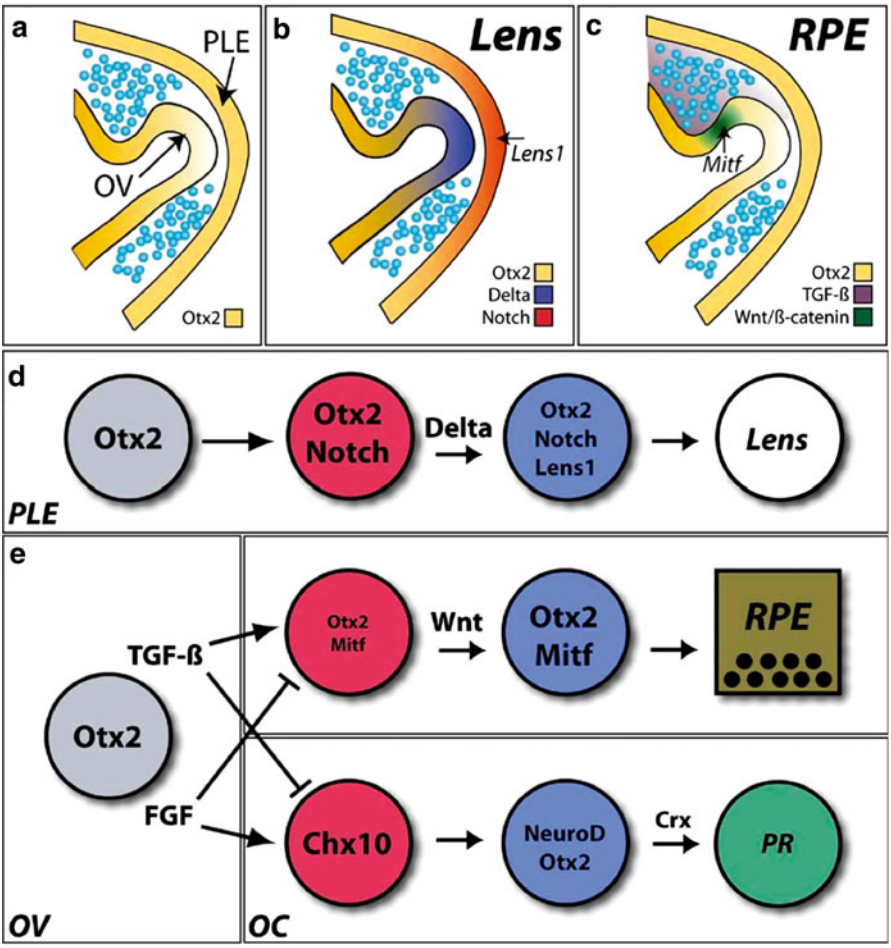

Fig. 1.1 Otx2 integrates with extrinsic and intrinsic factors to specify distinct cell-fates. (**a–e**) Depiction of Otx2 expression in the optic vesicle and the co-factors it associates with to induce lens, RPE, and photoreceptor fates. (**a**) Cartoon depicting Otx2 expression in the optic vesicle (OV) and presumptive lens ectoderm (PLE). Otx2 is downregulated in the presumptive neural retina prior to invagination. (**b**) During *Xenopus* lens development Otx2 integrates with extrinsic Notch/Delta signaling pathway members to activate *Lens1* in the PLE, a gene with multiple functions in lens development. Otx2 and Notch are expressed in the PLE, and Delta is expressed in the distal region of the OV. (**c**) During RPE development, TGF-β delivered from the extraocular mesenchyme specifies the RPE, and Otx2 integrates with Wnt/β-catenin signaling in presumptive RPE to induce *Mitf* and maintain cell-fate. (**d**, **e**) Cartoon depicting the step-wise processes involved to generate lens, RPE, and photoreceptors. (**d**) Depiction of the role of Otx2 during lens development. (**e**) Depiction of OV primordial cells specified by either TGF-β, which promotes RPE fate and *Mitf* expression and represses retina fate, and FGF, which promotes retina fate and perhaps activates *Chx10* and represses RPE fate. In the RPE (*top right*) *Otx2* integrates with Wnt/β-catenin signaling to enhance *Mitf* expression and to activate RPE differentiation genes. In the neural retina (*bottom right*) *Otx2* is induced in Chx10 expressing retinal progenitor cells and it interacts with NeuroD to activate photoreceptor cell differentiation genes such as *Crx*

observed in double *Pax6–/– Pax2–/–* mice including RPE-to-retina transdifferentiation. In single mutants, however, no RPE phenotype is observed implying that their redundant transcriptional activities are required [25]. The role of Pax6 in RPE differentiation was more clearly elucidated using chimeric *Pax6–/–* and wild-type mice. RPE differentiation is delayed in *Pax6* null cells, based on delayed activation of pigment genes and pigment synthesis, suggesting that *Pax6* may be important for regulating the rate or onset of RPE differentiation, but is not required [72, 73]. *Pax2* and *Pax6* were both reported to activate *Mitf-A* in vitro [25]. Since the deletion of both *Pax* genes results in a downregulation of all *Mitf* isoforms expressed in the RPE (and not just *Mitf-A*), it was suggested that *Mitf-A* induces expression of all the RPE-specific *Mitf* isoforms. However, *Pax2* and *Pax6* genes are downregulated before *Mitf* reaches its peak expression [3]. Furthermore, in *Otx2* mutants, *Pax2* and *Pax6* expression domains are ectopically expanded into presumptive RPE domains, but cannot prevent RPE-to-retina transdifferentiation [50]. Consequently, it was thought that they might only be involved in the initial activation of *Mitf* [3, 30].

A recent report has shown that Pax6 is utilized differently in RPE cells and in neural retina cells. According to a newly emerging model Pax6 operates in conjunction with *Mitf* and the RPE-specific Mitf paralog *Tfec* as an anti-retinogenic factor. When it collaborates with retinogenic genes including *Six6* and *Vsx2* (described below), however, it acts as a pro-retinogenic factor [48]. Understanding this molecular mechanism, and learning how to properly control Pax6 expression, may be useful for optimizing RPE and photoreceptor derivation techniques from pluripotent stem cells.

Visual System Homeobox 2 (Vsx2)

The *Vsx2* gene (formerly known as *Chx10*) contains homeodomain and CVC domains and has been demonstrated to directly inhibit *Mitf* [17, 74–76]. It is first detected in the distal optic vesicle (presumptive neural retina) of late optic vesicle stages and mutations result in microphthalmic eyes with *Mitf*-expressing pigmented retinas [17, 74–76]. Vsx2 forced expression in the mouse RPE interferes with pigment formation, but does not induce RPE-to-retina transdifferentiation [17].

A classic genetic experiment was performed by crossing *Mitf* (*mi/mi*) mutant mice, in which the RPE is ectopically hyperproliferating and expressing retina-specific markers, with Vsx2 mutant mice (*orJ*) that have hypoproliferating and pigmented retinas. In mice bred to homozygosity for mutations at both loci, the proliferative defects of the RPE and neural retina are partially corrected, suggesting that Vsx2 is a proretinal factor that represses *Mitf* [17, 77]. This was recently confirmed using molecular assays demonstrating that Vsx2 binds to *Mitf-D* and *-H* enhancers and represses their transcriptional activities [16]. This finding is significant since in *orJ* mutant retinas, expression of *Mitf-D* and *-H* transcripts are dramatically elevated, while the expression of *-A* and *-J* isoforms levels barely change. Additionally, crosses of *orJ* mutant mice with *Mitf-D* and *-H* deficient mice (*Mitf* (*mi-rw*)) partially correct the RPE and retina deficiencies [16]. This finding confirms that *Mitf-D* and *-H* have much more prominent roles in the RPE than *-A* or *-J*.

1.1.1.2 Extrinsic Regulators of Retina Development

Extrinsic factors secreted in paracrine or autocrine manners operate to induce and refine gene expression profiles and cell-fates of primordial optic vesicle cells. Extrinsic factors also reinforce cell-fate decisions of RPE cells. Extrinsic regulators of RPE differentiation include TGF-β family members, Hh proteins, FGFs, BMPs, and BDNF. Activin-like, Shh, and Wnt/β-catenin signaling can maintain RPE cell fate. A few of these are reviewed in detail and some others are summarized in Table 1.2.

Activin-Like Signaling

Activin-like signaling is required and sufficient to specify the RPE [15]. In chick optic vesicle explants in which mesenchymal cells have been removed, RPE markers such as Mitf, Wnt2b, and melanosomal matrix protein (MMP115), are not expressed and display expanded expression domains of retinal markers Vsx2, Pax6, and Optx2 [15]. The exogenous substitution of mesenchymal cells with Activin A, a TGF-β superfamily member, induces the expression of RPE markers thereby rescuing the defect [15]. In chicken, type IIA and IIB Activin receptors are expressed in the RPE in a sustained pattern of development, and key downstream effectors Smad2/3 proteins are phosphorylated in the RPE, showing that Activin signaling is active in the right place and time to induce cell-fate in the RPE [15, 78].

Activin βA is robustly expressed in the extraocular mesenchyme (and weakly in the RPE) during optic cup stages suggesting that it may be important for maintenance of RPE cell-fate [78]. In fact, the exogenous application of Activin to chicken and mouse explant cultures can prevent FGF-induced RPE-to-retina transdifferentiation, and the window in which transdifferentiation can be induced by FGF is extended when Activin signaling is pharmacologically inhibited [78]. These results strongly suggest that Activin signals are important for maintenance of RPE cell fate. However, it is not known if TGF-β pathway effector proteins directly regulate RPE-specific genes including *Mitf*.

Hh Signaling

Hedgehog proteins may specify the ventral RPE, be important for regulating *Otx2* and *Pax* genes, participate in the patterning of the optic vesicle, and be important for the maintenance of RPE cell-fate [19, 20, 26, 79, 80]. Several hedgehog proteins are expressed in the RPE of different organisms including sonic (*shh*) and tiggywinkle (*twhh*) in zebrafish; sonic (*X-shh*), banded (*X-bhh*), Indian (*X-Ihh*), and cephalic (*X-chh*) in *Xenopus*; and Indian (*Ihh*) in mouse [79, 81–84]. In *Xenopus*, tadpoles treated with an Shh antagonist, cylopamine, develop nonpigmented RPE [79]. In chicken, Shh-blocking antibodies also disrupt pigmentation, induce Otx2 downregulation in the ventral region, and induce RPE-to-retina transdifferentiation. The forced expression of Shh activates pigment synthesis and Otx2 expression in the retina [20].

Table 1.2 Extrinsic positive and negative regulators of RPE fate

Factor	Source/organisms tested	Function	Experimental perturbations
Activin-like	EOM of mouse and chicken [15]	Can activate *Mitf*, *Wnt2b*, and repress *Vsx2*. Important for OV patterning, specification, and maintenance of RPE fate [15, 78]	Removal of extraocular mesenchyme results in a failure to specify the RPE. Effect can be rescued by exogenously applying Activin A [15]
Shh	Ventral midline of mouse, chicken, frog, and zebrafish [201]	OV patterning, RPE specification, *Otx2* regulation [20]. Maintenance of RPE fate [80]	RPE-to-retina transdifferentiation induced in ventral RPE of chicken when inactivated and in BF1-deficient mice [19, 20]. Inactivation in frog induces pigment defects but not transdifferentiation [20, 79]
BDNF	Autocrine in frog [202]	Survival and differentiation of RPE [202]	BDNF inhibiting antibodies in frog prevent RPE differentiation [202]
BMP	Dorsal SE of chicken, presumptive RPE, EOM [12, 203]	Specification or maintenance of RPE fate [12, 203]	Microphthalmia and RPE-to optic stalk respecification occurs when BMP signaling is inactivated. BMP4 and 5 may be sufficient to induce RPE fate [12, 203]
FGF	Lens ectoderm, OV of mouse, and chicken [3, 30]	Potent neural retina inducer and RPE repressor. Important for OV patterning [18, 88, 97]	Induces RPE-to-retina transdifferentiation when activated in RPE domains. Removal of surface ectoderm in mouse and chicken results in a failure of neural retina specification [18, 88, 97]
Wnt	Active in the murine presumptive RPE [21, 22, 104, 106]	Required for maintenance of RPE fate. Regulates both Mitf and Otx2 by activating the *Mitf-D*, *Mitf-H*, *Otx2T0*, and *Otx2 FM2* enhancers [21, 22]. Otx2/β--catenin co-transfections induce Mitf expression in vivo [23]	Conditional ablation in embryonic RPE cells results in RPE to neural retina transdifferentiation [21, 22]. In vivo electroporation of β-catenin and Otx2 results in RPE fate induction in vivo [23]

OV optic vesicle, *EOM* extraocular mesenchyme, *SE* surface ectoderm

Since Shh is derived from the midline, it may be important for specifying the ventral RPE by regulating Otx2, although it is not clear if this occurs directly or indirectly [19, 20]. Additionally, in chick it has been demonstrated that Shh is important for maintaining RPE fate, as ectopic Shh can inhibit RPE-to-retina transdifferentiation induced by FGF [80].

Shh has also been demonstrated to upregulate Pax2 in the ventral optic vesicle, and repress Pax6 expression. This is a key event that helps to divide the presumptive neural retina and optic stalk domains. If Pax6 is misexpressed in Pax2 expressing domains of the optic stalk, optic stalk to RPE respecification can occur [25].

FGF Signaling

Classical and more recent findings suggest that signals emanating from the surface and lens ectoderm, later determined to be FGF1 and FGF2, promote neural retina development [18, 85–88]. Removal of the surface ectoderm in mouse or chicken dramatically interferes with neural retina development by promoting RPE-like fates, a defect rescued in vitro by substituting the ectoderm with either FGF1 or FGF2 [18, 87]. Inhibition of FGF2 in chicken also interferes with retina development. However, no gross ocular phenotype is observed in FGF1/FGF2 double knock-out mice suggesting redundancy [88, 89]. FGF3, FGF8, FGF9, and FGF15 (and FGF receptors) are expressed in the presumptive neural retina in the optic vesicle stages [90–95]. Furthermore, ectopic application of several FGF family members induces RPE-to-retina transdifferentiation in mouse and chicken [17, 18, 28, 29, 94–98]. Also, more direct mechanisms have been elucidated as Ras and MAP kinase-kinase (MEK), members of the Raf-MEK-mitogen-activated protein kinase (MAPK) signaling pathway, one of which through FGF proteins operate, induces transdifferentiation [95, 99].

The FGF signaling pathway may induce or operate with Vsx2. The exogenous addition of FGF2 to *orJ* mutant eyes does not induce RPE-to-retina transdifferentiation or Mitf repression [17]. Therefore, FGF molecules emanating from either the surface ectoderm or the presumptive neural retina acting through the MAPK pathway may activate *Vsx2*, which both promotes neural retina development and represses *Mitf*.

Wnt/β-Catenin signaling

Clues that Wnt/β-catenin may be involved in maintenance of RPE cell fate came from several observations that Wnt/β-catenin pathway members including Wnt ligands, frizzled receptors, and other pathway components are expressed in vertebrate RPE and in neighboring mesenchymal cells during development [15, 100–103]. The most convincing data are that transgenic Wnt activity reporters are active, and the Wnt/β-catenin targets Axin2 and Lef1 are expressed in the distal presumptive RPE [21, 22, 100, 104–110]. However, Wnt/β-catenin activity in mouse is

observed only in the most distal region of the presumptive RPE at time-points when Otx2 and Mitf are already present [111] and activity ceases once the mice reach adulthood [22]. This finding is significant since it implies that Wnt/β-catenin signaling may be important for RPE differentiation and cell-fate maintenance, but not for specification.

The conditional inactivation of Wnt/β-catenin signaling in mouse and chicken RPE induces RPE to transdifferentiate into retinal neurons. In mouse, cre-mediated recombination of β-catenin after RPE-specification induces rapid *Mitf* and *Otx2* cell-autonomous downregulation and RPE-to-retina transdifferentiation [22]. This is also observed in chicken when the pathway is inactivated by misexpression of *dominant-negative Lef1* in the RPE [23]. Chromatin immunoprecipitation (ChIP) and luciferase assays indicate that β-catenin binds near to potential binding sites and activates the *Mitf-D* and *Otx2T0* enhancers [22, 23]. The Wnt/β-catenin pathway also activates the *Mitf-H* and *Otx2 FM2* enhancers in vitro [21]. This is significant since no other pro-RPE exogenous signaling factor has been shown to activate both *Mitf* and *Otx2*.

The Wnt/β-catenin pathway does not appear to function significantly in the neural retina, however. In chicken and zebrafish transgenic reporter lines, essentially no activity is observed in the central retina [100, 105, 107]. In mouse, however, TCF/LEF activity is observed, but only in select lines, and the activity observed in the central retina of at least one of these lines (TOPgal) may occur in a Wnt/β-catenin-independent manner since activity persists after β-catenin deletion [103, 106, 110, 112]. Furthermore, the conditional inactivation of the Wnt/β-catenin pathway in the neural retina results in dramatic lamination defects, but cell-fate is not affected [113]. The lamination defects are induced through the loss of cell–cell contacts by depleting β-catenin that localizes in adherens junctions [114].

1.1.2 Maintenance of Cell Fate and Terminal Differentiation

RPE fate is not absolute, and several reports have shown that the RPE fate is plastic in species-specific temporal windows. RPE-promoting extrinsic factors including Activin-like, Shh, and Wnt/β-catenin signaling have been shown convincingly to be important for the maintenance of RPE-fate [15, 21, 22, 78]. When mesenchymal cells are removed from chick optic vesicles and maintained in culture, *Mitf* is only induced when Activin A is added, implying that an Activin-like factor secreted by extraocular mesenchymal cells induces RPE cell fate [15]. Similar to observed Wnt/β-catenin signaling activities, both Activin ligands and receptors are expressed at the right time and place to provide constant cell-fate reinforcement. Activin βA is consistently expressed in high amounts in the mesenchyme and low amounts in the RPE during optic cup stages, and Type IIA and IIB Activin receptors are continuously expressed in the RPE [15, 78]. Inhibiting Activin/TGFβ/nodal signaling can extend the window of RPE competence to generate neural retina. Conversely, the exogenous application of Activin A can prevent FGF-induced transdifferentiation,

strongly suggesting also that Activin A is a potent reinforcer of RPE fate [78]. Likewise, ectopic Shh delivery inhibits FGF induced RPE-to-retina transdifferentiation and Hh inhibition increases the transdifferentiation domain, also suggesting Shh signaling is also important for the maintenance of RPE fate [80].

The extrinsic factors described above all converge on *Mitf*, *Otx2*, and *Pax6*. Not only is it important that each gene is expressed at the right time and place, but the gene dosage appears to be critical as well based on the following pieces of evidence. When two homozygous transgenic *Mitf* alleles are combined in *Vsx2* mutant mouse retinas, a pigmented monolayer is generated from the neural retina. Ectopic *Mitf* is spontaneously upregulated in the retina of these mutants but the *Mitf* expressing cells resemble RPE/neural retina intermediate cells instead of RPE monolayers suggesting that higher doses of Mitf can induce more RPE characteristics [17]. We have shown that the level of Mitf expression is significant in the RPE and retina, and that when Mitf levels are increased, more pronounced RPE characteristics are observed. While *Mitf* cannot be induced in sufficient levels to induce ectopic pigment synthesis in the chicken retina with a combination of *CA-β-catenin* and *Otx2* through E3.5 stages, direct overexpression of *Mitf-D* is sufficient in a few cells to induce ectopic pigmentation in the retina and RPE by E3.5, and MMP115 is induced in low numbers of scattered cells [23]. Similar effects were reported when chicken *Mitf* is overexpressed in the chicken retina [115]. In gain-of-function *Mitf* mutants, pigment was observed sporadically in transfected cells and the direct *Mitf* target gene *Dct* is ectopically upregulated, albeit also in scattered cells [23]. Additionally, the *Mitf* gain-of-function mutant retina proliferates more slowly than controls, and expression of the cell-cycle inhibitor p27 (kip1) is enhanced, thereby more closely matching the proliferation rates of the RPE, not the retina [115]. In transgenic mice lacking *Mitf-D*, total levels of *Mitf* are attenuated at E11.5, but normal levels are restored at E13.5 through compensatory upregulation of other isoforms. Intriguingly, the only time a noticeable phenotype is observed in the *Mitf-D* knockouts is E11.5 when pigment defects and attenuated *Tyrosinase* expression is observed [48].

We have shown two independent mechanisms through which *Mitf* expression can be enhanced. First, *Mitf-D* is capable of directly autoregulating its own enhancer to regulate its own expression levels. The second mechanism is through the combined transcriptional activities of Wnt/β-catenin signaling and *Otx2*, which induce ectopic Mitf expression in vivo, even though neither *constitutively active-β-catenin* nor *Otx2* are sufficient alone to do so [22, 23].

Dosage of Otx2 may also be important for promoting RPE cell-fate. A higher percentage (35 %) of ectopic *Mitf* expressing cells are induced in cells in which Otx2 is robustly expressed. This fits well with biochemical data demonstrating that β-catenin and Otx2 may activate *Mitf-D* in an additive manner. Otx2 may also directly autoregulate the *OtxT0* enhancer [23]. This is important since the precise expression levels of *Otx2* may determine if the RPE develops properly or undergoes RPE-to-retina transdifferentiation.

The gene dosage of Pax6 has also been shown to regulate the transdifferentiation potential of RPE to neural retina in *Mitf* mutant mice. The mutation of one Pax6 allele in Mitf mutant mice exacerbates RPE to neural retina transdifferentiation,

and genetic-gain-of-function assays of Pax6 prevent the transdifferentiation. Furthermore, the combined activities of Pax6 with Mitf exert anti-retinogenic influences that prevent RPE to neural retina transdifferentiation [48].

1.1.3 Step-Wise Model of RPE Development

I propose that RPE-specification occurs in a step-wise manner, beginning with specification and the induction of *Mitf*. Once Wnt/β-catenin signaling is activated in the optic vesicle, it may operate through Otx2 to enhance *Mitf* expression levels, and eventually all three factors will begin to integrate their transcriptional activities, thereby resulting in a significant enhancement of RPE-specific gene expression.

The RPE is specified in the optic vesicle probably through Activin-like factors secreted from overlying extraocular tissues, and by Shh that emanates from midline tissues [15, 20]. Once Mitf is induced in the presumptive RPE, it may interact with members of the Wnt/β-catenin pathway to enhance *Mitf* target gene activation.

Lastly, Otx2 and Pax6 interact with Mitf to enhance their transcriptional abilities. In the neural retina a general pattern of differentiation has been described involving multiple transcription factors and homeobox transcription factors confer positional identities, while bHLH transcription factors activate transcriptional differentiation programs [70]. Accordingly, when *Otx2* and *Mitf* (bHLH) are co-transfected in luciferase assays, a synergistic enhancement of RPE-specific enhancer activation is observed. Furthermore, Otx2 and Mitf have been demonstrated to physically interact in the nuclei of transfected quail RPE cells [45]. Pax6 operates in conjunction with *Mitf* and *Tfec* to prevent retinogenesis during the critical period when RPE to neural retina transdifferentiation is possible in mice.

1.1.4 Co-dependence of RPE for Photoreceptor Development

Based on landmark genetic ablation experiments it was determined that the RPE is required to guide several steps of neural retina development [116]. RPE conditional ablation in early optic vesicle stages results in severely retarded growth of the retina and eye reabsorption. Variable results are seen when RPE are ablated in the optic cup stages that include retina lamination defects and vitreous defects, microphthalmia (small eyes), or anophthalmia (no eyes) occurs [116].

Indeed, the results of several in vitro and in vivo experiments demonstrate that the RPE is required to pattern and organize the retina. Avian and hamster retinal cells were shown to aggregate in culture but present severe lamination defects. These defects were rescued by introducing RPE cells or RPE conditioned media into the cultures [117–120]. In zebrafish, *N-cadherin*, *PKC*γ, and *mosaic eyes* (*moe*) mutants with RPE defects exhibit dramatic neural retina lamination defects and RPE defects [121–126].

Perturbations of single genes in RPE cells during development can also induce dramatic defects to the entire eye. Mutations to the pro-RPE genes *Mitf*, *Pax6*, and *Otx2* are also associated with microphthalmia and aniridia (for review see [14]). The conditional deletion of Vhl in embryonic RPE cells also results in microphthalmia and aniridia, and gross disturbances are observed in the ocular circulatory system (described below) [5]. The inducible deletion of vascular endothelial growth factor (VEGF) in adult RPE cells induces rapid collapse of the choriocapillaris and gross dysfunction of cone photoreceptors [127].

Finally, in humans death or dysfunction of RPE cells are characteristic of AMD, the most common cause of vision loss in industrialized countries.

1.1.5 Development of the Neural Retina

Many cases of retinal degeneration, including pre-AMD, are amenable to RPE-based transplantation therapeutic interventions (for review see [11, 128]). In cases of geographic atrophy (advanced AMD) RPE transplantation will provide no real effect for preserving vision since the photoreceptors have already degenerated. While inherently more difficult, only photoreceptor replacement may provide therapeutic benefit in these patients and in those with other advanced degenerative diseases. There is some precedence for this strategy, as retinal precursors can be successfully delivered to the outer retina and can restore visual behaviors in mice [129–132]. The main focus of this subsection will be centered on photoreceptor development to reinforce the challenges involving their derivation and use. The development of the entire sensory retina will be briefly discussed as well since work is underway to derive other cell types including ganglion cells.

Before discussing and outlining the steps involved in building the functional architecture of the sensory retina, it is important to define some key terms. These include potency, competence, and commitment. The *potency* of a precursor cell is defined by how many different cell-types can be generated from it. Pluripotent stem cells by definition should be able to generate any cell-type in the body. Pluripotent stem cells, however, are unlikely to exist in the eye. All the retinal cell-types are instead derived from multipotent progenitor cells with far more limited potentials. The potency of RPCs can vary depending on their environments. Importantly, extrinsic signals can dictate which cell-types are produced at any given time, but cannot cause RPCs to produce cell-types *not* ordinarily generated during that time frame. Based on these observations and others, the competence model emerged [133]. According to this model RPCs progress through different states of *competence* that are intrinsically defined. The results of landmark experiments have shown that precursor cells are pre-determined to make specific a cell-type(s) and will continue to generate them, even if incorporated into the retina at a time when these cells are not normally born [134–136]. Finally, *commitment* is used to define a recently defined cell-type that is fully determined and no longer responsive to extrinsic cues.

Table 1.3 Birth order and intrinsic regulators of retinal cell development

Cell-type	Birth order	Intrinsic factors
Ganglion cells	1	Atoh7, Pou41/2/3, Isl1, NeuroD1
Horizontal cells	2	Foxn4, Six3, NeuroD4, Ptf1a, Prox1
Cone photoreceptors	3	NeuroD1, Ascl1, Otx2, RORβ, Prdm1, Sall3, Pias3, Thrb, Rxrg, Rora, Nr2f1/2
Amacrine cells	4	Six3, Foxn4, NeuroD1, NeuroD4, Ptf1a, Bhlhb5, Isl1, Nr4a2, NeuroD6, Satb2, NeuroD2
Rod photoreceptors	5	NeuroD1, Ascl1, Otx2, Nrl, Nr2e3, RORβ, Prdm1, Pias3
Bipolar cells	6	Vsx2, NeuroD4, Ascl1, Bhlhb5, Vsx1, Irx5, Bhlhb4
Mueller glia	7	Rax, Hes1, Hes5, Hesr2

During development, the inner layer of the optic cup will become the neural retina and multipotent RPCs will generate all of the retinal cell-types in a largely evolutionarily conserved sequence [67]. Retinal cells are born in a well-characterized and stereotypical birth order as demonstrated using lineage tracing [35] and differentiate following a general pattern in which homeobox transcription factors that provide spatial cues interact with bHLH transcription factors to activate cell-type-specific transcriptional differentiation programs [70]. The ganglion cells are born first, then amacrine cells, cone photoreceptors, horizontal cells, and finally bipolar cells, rod photoreceptors, and Mueller glia. After occupying specific layers of the developing retina, the neurons will undergo terminal differentiation and initiate synaptogenesis as they become terminally differentiated. The final commitment steps of RPCs can also be a complicated process. In some cases genes are activated that are responsible for repressing development of other cell fates. One notable example, that will be described in more detail shortly, is the transcriptional dominance model of rod vs. cone fate determination [137].

How carefully then must the competence model be recapitulated in vitro in order to generate viable retinal cells for transplantation? In other words, must pluripotent stem cells be first strictly guided into multipotent RPCs, guided into photoreceptor precursors, and then differentiated into rods and cones? Or could the intermediate steps be safely skipped? How much do we need to know about what extrinsic signals are involved, how important are they, and when should they be delivered to mimic developmental steps in vitro? And how important is it to identify distinct intrinsic transcriptomic fingerprints across multiple time-points that define their competence? Finally, the more we understand about terminal differentiation of specific cell-types, and as we gain access to complete gene expression profiles of differentiated cells, the more confidence we can have utilizing them for replacement therapies.

We do not know how all of the retinal cell-types are specified, and many of the neurogenic gene networks are very complicated. However, many intrinsic factors have been identified and a short list is provided in Table 1.3. There are many key questions left to address, mainly how are the intrinsic factors of a progenitor cell regulated to convince it become one cell-type, while another progenitor cell that was born at the same time will generate a completely different cell-type?

The answer may lie in the observation that RPCs are remarkably heterogeneous [138], thus allowing for some flexibility, although other explanations must also be considered.

Just as there is considerable overlap in the sequence in which different retinal cells are generated, some of the intrinsic factors employed can be used to generate more than one cell-type. NeuroD is a commonly employed gene that is expressed in multiple ocular cell types at different times during development; it has been implicated in cell fate determination, retinal cell differentiation, and neuron survival [139, 140]. The dual roles of Pax6 to generate both RPE and neural retinal cells have already been discussed, as have the diverse functions of Otx2 in eye development. Therefore, when characterizing a cell-type generated from stem cells it may not be enough to show that a gene is expressed but that it is expressed at the right time (and that the proper co-factors are present).

Photoreceptor development has been well studied, and many of the intrinsic and extrinsic factors regulating their differentiation have been identified [137]. Rods and cones are generated in unequal ratios (greatly favoring rods) and spaced in non-random mosaic patterns. In some animals, including humans, a cone-dense macula develops that allows for acute central vision, and the type of opsin proteins they synthesize can distinguish cone subtypes. Short (S-), medium (M-), and long wavelength-sensitive opsins (L-opsins) are synthesized in cones, and rhodopsin is made in rods. The three different opsins in cones provide humans with trichromatic vision. (Mice only have dichromatic vision since they only generate only S- and M-opsin containing cones.)

Photoreceptor differentiation is a time-consuming process [141, 142]. RPCs are converted to photoreceptor precursors in a manner largely controlled by Notch signaling and the bHLH transcription factors HES1 and HES5 [143, 144]. Rod and cone precursors express opsins in early steps of development but remain immature for several weeks in humans. S-opsin is generated first (S-opsin expressing cones are generated unless instructed otherwise); M-, L-opsin, and rhodopsin are detected shortly afterwards [145]. Expression of all of the opsins increases steadily as outer segment biogenesis proceeds. During the final steps of photoreceptor development the photoreceptors begin to make functional synapses and initiate phototransduction.

1.1.5.1 Intrinsic Regulators of Photoreceptor Development

Development of rods and cones from photoreceptor precursors occurs largely by a complex interplay of genes including, but not limited to *Otx2*, *Crx*, and *Nrl*. Some of the promiscuous roles of Otx2 in eye development have been addressed previously already. Its role in photoreceptor development cannot be disputed since the conditional loss of function in murine RPCs results in formation of retinas that are nearly completely devoid of photoreceptors [54, 146]. Just as Otx2 requires a co-factor to prime RPE fate, Otx2 likely requires a co-factor (likely *Mash1* and the dual regulator of rod and cone differentiation *RORβ*) for photoreceptor development [54]. Otx2 also functions in photoreceptor precursors to activate expression of *Crx* [147],

a gene linked to multiple diverse and clinical phenotypes including cone-rod dystrophy, retinitis pigmentosa, and Leber Congenital Amaurosis [148–152]. *Crx* is responsible for activating a host of key photoreceptor genes, and is required for photoreceptor terminal differentiation. In fact the loss of *Crx* function induces the formation of photoreceptors that remain in immature states and eventually degenerate [153]. Photoreceptor precursors become rods or cones due to the activity of *Nrl*. The loss of *Nrl* in mice results in rod-deficient retinas that are instead dominated by S-opsin expressing cones [154]. *Nrl* can also be instructive as transgenic gain-of-function experiments demonstrate that it can direct RPCs into rod fates [155]. Finally, Nrl interacts with Crx to activate rod photoreceptor-specific gene expression including *Nr2E3* (suppressor of cone fate) [154–156].

1.1.6 Step-Wise Model of Photoreceptor Development

A step-wise model of murine photoreceptor development based on transcriptional dominance is as follows (for review see [137]). RPCs are converted to rod and cone photoreceptor precursors by Otx2. Otx2 associates with RORβ and Crx in the precursors to activate photoreceptor-specific gene expression. The photoreceptor precursors will, by default, become S cones unless they are exposed to additional regulatory signals that redirect them into M or rod fates. The induction of Nrl and NR2E3 induce rod fate (and suppress cone fate). M cones are generated at the expense of S cones by TRβ2.

1.1.7 Development of the Vasculature

The earliest source of oxygen and nutrients for the developing eye is from a transient vascular network, the hyaloidal vessels that regress concomitant with development of the retinal vasculature [157–159]. Others and we have shown that both processes are likely orchestrated by a combination of retinal neurons, astrocytes, and macrophages in a manner that is at least in part controlled by oxygen availability [5, 157, 158, 160–162]. As the neural retinal cells proliferate, differentiate, and mature, oxygen demands are altered. Insufficient oxygen availability results in activation of the hypoxia-inducible transcription factors (HIFs). HIFs activate a host of genes that promote cell survival and activate angiogenesis [163–165]. During normoxia HIFs are rapidly and efficiently degraded by pVHL to prevent ectopic HIF stabilization [166]. In many species including mice, rats, and humans three plexus layers form between the retinal neurons (for review see [167]). The manner in which they form has yet to be determined, although VHL/HIF signaling in retinal neurons and RPE cells exert significant roles, and VEGF and Ras signaling are certainly involved [168–171].

Persistence of the hyaloidal vessels and grossly disorganized plexus layers are observed in mice in which Vhl is conditionally ablated in retinal neurons or RPE cells

during embryonic stages [5, 157]. Other severe abnormalities are also induced when Vhl is deleted in embryonic RPE cells including small eyes (microphthalmia), aniridia, and apoptosis of photoreceptors cells [5]. RPE cells are also responsible for development of the choroidal vasculature during embryogenesis [4, 6, 7], and continue to provide essential vasculotrophic support in adult stages. We have shown that RPE-derived VEGF is required for maintenance of the choroidal vasculature; just 3 days after the inducible deletion of VEGF in the RPE near complete collapse of the choriocapillaris is observed and cone dysfunction is measurable just 7 days post-ablation [127]. Maintenance of the choroidal vasculature is paramount, thinning of the choriocapillaris is characteristic of AMD [172, 173], and choroidal neovascularization is characteristic of "wet" or neovascular AMD. Vessel attenuation is a common outcome in cases of retinitis pigmentosa [174] and neovascularization is seen in cases of Retinal Angiomatous Proliferation (RAP) and Macular Telangiectasia (MacTel).

Integrity and stability of the vasculature must be considered when any cell transplantation techniques are employed. Hyperoxia is believed to be a strong stimulus that exacerbates photoreceptor degeneration [175]. Implanting stem cell-derived cells into hyperoxic or hypoxic environments not only might limit their survival potential, but also may alter oxygen availability for neighboring cells and induce detrimental stress responses. Successful implantation techniques therefore may require rebuilding or stabilizing the vasculature.

1.1.8 Generation of Bruch's Membrane

Bruch's membrane is a collagen and elastin-dense extracellular matrix generated by RPE, endothelial cells in the choroid, and perhaps invading fibroblasts during eye development [32, 176]. Interestingly, RPE cells begin to activate some of the extracellular matrix genes to build Bruch's membrane during a specific window of photoreceptor maturation [32]. Bruch's membrane is the only separation between the RPE and choriocapillaris. For nutrients and oxygen to reach the highly demanding photoreceptors, they must travel through fenestrations in the choriocapillaris, passively diffuse across Bruch's membrane, and be transported by RPE cells into the subretinal space. Thickening of Bruch's membrane with lipid-rich linear deposits may promote closure of the choriocapillaris and photoreceptor cell atrophy [177]. A strong correlation was demonstrated between thickening of Bruch's membrane and the presence of ghost vessels in the choriocapillaris of AMD patients [173]. Focal atrophy of the choriocapillaris can limit oxygen and nutrient supply to photoreceptors and promote dysfunction or death [172].

RPE transplantation strategies may be greatly limited by the integrity of Bruch's membrane. It is unclear if implanted RPE cells can "rebuild" or repair Bruch's membrane, so other options such as "cleaning" diseased Bruch's membrane with detergents that may be equally challenging, but perhaps feasible, are being explored [178]. Efforts are also underway to culture RPE monolayers on polymers for transplantation that can support or replace diseased Bruch's membrane [179].

1.1.9 Retinal Remodeling

Alterations to the integrity of the ocular vasculature and to Bruch's membrane are associated with aging and common features associated with AMD. Besides considering the status of these structures prior to cell transplantation, another important consideration is stress-induced retinal remodeling (for reviews see [180, 181]). Once photoreceptors become stressed a host of non-cell autonomous defects can be induced. Initially photoreceptors begin to uncouple and start forming new synaptic connections. As photoreceptors succumb to stress, Mueller glia become adherently activated and form glial scars; RPE cells can become hypertrophic and migratory. Eventually remodeling occurs in all retinal neurons, which is significant since even minor changes have the potential to severely convolute signal processing. While an optimistic view may be that implanted RPE or photoreceptor cells could reverse many of the remodeling changes, the chances of this occurring seem extremely remote. Therefore the degree of retinal remodeling should be considered when cell transplantation-based therapies are being considered since implanted cells would not only need to form proper synapses with appropriate neighboring cells, but also undo much of the damage that all evidence suggests is likely irreversible.

1.1.10 Lessons from Nature

What lessons can we learn from nature that can guide strategies for deriving specific cell-types from stem cells? A few important questions might be asked.

1. Is it imperative to precisely recapitulate development steps that occur in vivo when deriving specific cell-types? Will an exact recapitulation actually result in more valid or safer cells? Is it worth the added expense to do so, and might introducing exogenous factors, many of which are made in E. coli cells, actually promote enhanced immunogenicity?

   The answer to these questions may be that the developmental steps eye progenitors proceed through in vivo to build an entire eye are unnecessarily complicated. To generate an eye, gradients of often opposing factors exist that force developing cells to activate genes that repress opposing fates. Maintenance of cell fate, perhaps especially for RPE cells, is a strict requirement in vivo but could perhaps be bypassed in vitro.

   Evidence to support this claim comes from the fact that the conversion of iPS or ES to photoreceptors or RPE cells either requires a very minimal cocktail of signaling factors or, in the case of RPE, occurs spontaneously (albeit at low yields) in culture. This does not mean that recapitulating developmental pathways provides no benefit, however. Photoreceptor derivation has been optimized through the use of important extrinsic factors. Photoreceptors have been generated from hES cells in neural differentiation media using combinations of IGF1, noggin, LEFTY, and DKK1 [182–186]. To make RPE cells, others and we have shown that supplementing differentiation media with Nicotinamide and Activin A to the RPE

cultures increases yield and accelerates the derivation time [187, 188]. Two groups have demonstrated that the process can be dramatically accelerated using combinations of eye field transcription factors, exogenous factors known to induce neural retina progenitor fate, and pro-RPE factors. RPE can differentiate from stem cells at an incredible rate (14 days) by adding Nicotinamide, IGF1, Noggin, Dkk1, and bFGF to first convert the stem cells to neural retinal progenitor fates, and then by supplementing the media with pro-RPE factors including nicotinamide and Activin A [189]. Another recent report showed that RPE can be derived directly from fibroblasts in roughly 1 month by transducing them with a minimal set of transcription factors that include cMyc, Mitf, Otx2, Rax, and Crx [190]. It is noteworthy that neither of these derived lines was rigorously characterized nor was it implanted in vivo to demonstrate full functionality. Thus it remains an open question if they are in fact as good as cells derived using conventional techniques. Therefore, while it is not necessary to strictly recapitulate developmental pathways in order to generate photoreceptors and RPE cells, simplified protocols involving only the most critical factors can be used to accelerate and perhaps optimize the derivation process.

2. What unexpected effects might be induced through the manipulation of gene networks either to induce pluripotency in somatic cells or to directly convert fibroblasts to RPE?

   There is evidence that genetic manipulations to induce pluripotency can affect the differentiation potential, tumorigenicity, and immunogenicity of induced pluripotent stem cells (iPS) cells. We have shown RPE derived from iPS reprogrammed using a minimal set of viral-induced transcription factors (Oct4 only with small molecules) rather than a viral-induced full set of Thomson factors (Oct4, Sox2, Nanog, and Lin28) most closely resembled human fetal RPE based on metabolomic analyses [188]. Comparative analyses of multiple human iPSC (hiPSC) and human embryonic stem cell (hESC) lines reveal that although many share very similar transcriptomic and epigenetic profiles, others are heterogeneous, and this can limit their differentiation potential [191]. Furthermore, reprogramming and selection pressure to obtain rapidly proliferating cell lines may induce chromosomal aneuploidy in nonrandomly distributed loci that can limit their differentiation capacities and promote tumorigenicity [192–194]. Finally, reprogramming somatic cells to iPS may activate gene networks that actually promote heightened immunogenicity [195]. Therefore, it is important to understand exactly what effects the genetic manipulation of molecular networks can have on cells as many of these effects may linger even after terminal differentiation.

3. Are current stem cell-based therapies truly feasible in human subjects based on several of the considerations covered in this review? Will neural retina cells integrate into diseased retinas appropriately, not just to restore light responsiveness, but also to generate accurate visual fields (especially since the retina is prone to remodeling). Will implanted RPE cells adhere properly on diseased Bruch's membranes? If porous substrates are used as culture supports for RPE cells, will these allow for proper ion and water exchange between the RPE and choriocapillaris? These are open questions that we may not be able to answer until the clinical trials that are underway have been completed.

Besides the major questions presented above, there are other considerations as well. One concept that has not been addressed in this review is that of generating the wide array of retinal cell subtypes. For photoreceptors and RPE cells this is not a major concern since there are just four types of photoreceptors in humans, and no RPE subtypes have been identified (although RPE can be very heterogeneous in shape, pigmentation, and function [196]). For derivation of ganglion cells, however, many subtypes have been identified; these localize in different regions of the ganglion cell layer and perform discrete functions.

The last consideration is if intact grafts could be generated for transplantation. There are potentially many different variants to consider including RPE/photoreceptor grafts, Bruch's membrane/RPE/photoreceptor grafts (or RPE/photoreceptor grafts grown on substrates), or actually entire grafts that comprise an entire section of intact retina. Derivation of both RPE and photoreceptors may be dramatically improved through co-culture, and intact human eyecups have been generated in vitro. However, the cell-types in the eyecups do not fully maturate and lack supporting tissues including astrocytes, microglia, and vasculature. Implantation of large grafts could also be quite complicated.

1.1.11 Summary

Development of the sensory retina proceeds as multipotent progenitor cells pass through different competence states to form the retinal neurons, glia, and RPE cells. RPE cells direct many steps of eye development, the vasculature, and Bruch's membrane. Defects in these structures or RPE cells are characteristic of AMD. While no cure exists for AMD, encouraging evidence suggests that replacement of diseased RPE with healthy stem cell-derived RPE may prevent photoreceptor degeneration. In cases of advanced retinal degenerations, the implantation of stem cell-derived photoreceptors may restore some visual behaviors.

These therapeutic approaches are guided by lessons we learn from embryonic development of these cells. By elucidating the molecular networks and identifying expression patterns and functions of critical intrinsic and extrinsic factors, we will be able to optimize derivation protocols. Finally, understanding how the microenvironments of retinal cells and how they are formed in vivo will provide insights for what may be required for implanted cells to full integrate and function properly. Therefore, as we build a stronger knowledge base of eye development, we can utilize this information to optimize and develop more effective therapeutic interventions.

Acknowledgments I would like to acknowledge the incredible help and guidance of my graduate thesis advisor Sabine Fuhrmann at the Moran Eye Center. In fact some portions of this work were presented in thesis form in fulfillment of the requirements for the PhD for Peter Westenskow from the University of Utah. I also wish to thank my postdoc advisor Martin Friedlander at The Scripps Research Institute for allowing me to take time away from my lab responsibilities to write this chapter. Finally, I would like to acknowledge the National Eye Institute (EY021416) for funding my research.

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Chapter 2
Mitochondria in Retinal Neurodegeneration and Stem Cell Models

Ian A. Trounce

Contents

Abbreviations

ADOA	Autosomal dominant optic atrophy
AMD	Age-related macular degeneration
ATP	Adenosine triphosphate
CMT	Charcot–Marie–Tooth
CPEO	Chronic progressive external ophthalmoplegia
ESCs	Embryonic stem cells
GWAS	Genome wide association studies

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A. Pébay (ed.), *Regenerative Biology of the Eye*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4939-0787-8_2, © Springer Science+Business Media New York 2014

iPSCs	Induced pluripotent stem cells
KSS	Kearns–Sayre syndrome
LHON	Leber hereditary optic neuropathy
MELAS	Mitochondrial encephalopathy lactic acidosis and stroke-like episodes
mtDNA	Mitochondrial DNA
NAD	Nicotinamide adenine dinucleotide (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
NARP	Neurogenic atrophy and retinitis pigmentosa syndrome
OXPHOS	Oxidative phosphorylation
POAG	Primary open angle glaucoma
RGCs	Retinal ganglion cells
RPE	Retinal pigment epithelium
TIM	Translocase of the inner membrane
TOM	Translocase of the outer membrane

2.1 Introduction

Defective mitochondrial energy production is an underlying cause of many rare genetic diseases that result in vision loss and is increasingly implicated in the common age-related retinal diseases—age-related macular degeneration and glaucoma. Mitochondrial energetic impairment results in disease in tissues that have the highest metabolic demands, including the central nervous system, cardiac and skeletal muscles, kidneys, and endocrine organs. Visual impairment is a common feature of these multisystem diseases such that study of retinal neuronal impairment is therefore a key model system to understand pathogenesis and therapeutics that has wide implications in both genetic disease and sporadic age-related degenerative conditions.

The mitochondrion is increasingly recognized as a key organelle in the integration of cellular signalling; not only metabolic regulation but also for decisions on cellular fate including maintenance of pluripotency and differentiation of progenitor cells, and programmed cell death. These different strands of mitochondrial biology are reviewed in this chapter with an emphasis on the unique genetics of the organelle, the key ATP producing pathway of oxidative phosphorylation (OXPHOS), and implications for retinal neuronal health and stem cell approaches to modelling and therapeutics.

2.2 Mitochondrial Genetics and Metabolism: Endosymbiotic Heritage

The mitochondrial genome (mtDNA) is the genetic remnant of the endosymbiotic event that gave rise to eukaryotes [1, 2]; it has profound and underappreciated implications for molecular biology, genetics, and medicine. It exists in hundreds to thousands of copies per cell, is maternally inherited in animals, and appears to lack homologous recombination making it technically challenging to manipulate experimentally.

The 13 protein genes remaining in this vestigial intron-less genome remain crucial to cellular viability because they encode core subunits of the OXPHOS complexes I (7 of 44 subunits), III (1 of 11 subunits), IV (3 of 14 subunits), and V (2 of 19 subunits) [3, 4]. In addition to these 13 protein-coding genes, the mtDNA encodes 2 rRNAs and 22 tRNAs needed for translation of the mtDNA-encoded proteins. This translation apparatus is semiautonomous to the nuclear system, using a different genetic code and being sensitive to different antibiotics to the cytoplasmic translation system such as chloramphenicol, betraying its bacterial ancestry.

With evolution, most of the genes encoding the metabolic machinery of the mitochondrion have been transferred to the nuclear genome, including all those required for the Krebs cycle, mitochondrial fatty acid oxidation, uridine synthesis, and many more, perhaps 1,500 gene products in total. The question therefore arises as to why the mtDNA persisted, and consideration of this mystery may provide insights to help overcome what has clearly become a crisis in modern biomedicine—that progress in genetic discovery and curative therapeutics has slowed despite massively increased resources. This concept has been developed and eloquently argued by Wallace, and the interested reader is referred to his recent reviews and perspective pieces [4–6]. In essence, Wallace argues that key energetic genes remained encoded in the organelle genome because while speciation may depend on nuclear gene mutation, metabolic adaptation of populations is driven by the more rapidly evolving mtDNA. One consequence is that coevolving nuclear genes of the OXPHOS pathway will have geographic signatures and combination of large numbers of subjects from different regions in genome-wide association studies (GWAS) will lose such signals.

An emerging concept of mitochondrial metabolic signalling is the control exerted by mitochondrial oxidative metabolism on nuclear gene regulation via epigenetic modification of histones (acetylation/deacetylation, phosphorylation) and DNA (methylation). The metabolic signals of nutrient abundance became ATP, acetyl-CoA, and the NADH/NAD ratio, all controlled by the level of OXPHOS-driven ATP production [6].

With the symbiosis begun by the engulfment by a glycolytic bacterium of an aerobic bacterium—the mitochondrial ancestor—regulation of these two central ATP-generating pathways enabled the eventual evolution of differentiated cells. This has important implications for both neuronal diseases including retinal disease, and also for the regulation of pluripotency. Here the involvement of mitochondria in retinal disease, and stem cell modelling of these diseases, is reviewed before briefly examining the growing evidence of mitochondrial control of pluripotency and differentiation.

2.3 Mitochondrial Dysfunction in Degenerative Retinal Diseases

Neurons use large amounts of ATP supplied by mitochondria. Energetic needs are greatest at unmyelinated regions including dendrites and synapses where ATP-dependent ion pumping reinstates the plasma membrane electrical potential consequent to impulse

transmission [7]. The purposeful transport of mitochondria along axons to the sites of ATP usage is also an energetic process. Kinesin moves mitochondria in the anterograde direction; whereas retrograde transport is orchestrated by dynein motors [8]. Mitochondrial diseases can present with complex multisystem pathologies, but central nervous system signs are most common [3, 9]. Retinal ganglion cells (RGCs, the axons of which form the optic nerve), auditory ganglion cells, and peripheral nerves are very commonly affected. A common feature of these neurons is that they have extremely long axons. The combination of higher energetic requirements for longer axons and the greater reliance on mitochondrial trafficking in these structurally complex cells may be a reason for their susceptibility to energetic compromise.

2.3.1 Primary Optic Neuropathies

Leber's hereditary optic neuropathy (LHON) is the prototypic mitochondrial disease since it was the first human disease proven to result from mutation of the maternally inherited mtDNA [10]. LHON is characterized by the specific loss of RGCs and is one of the most common mtDNA-linked diseases. Most cases (~90 %) are due to mutations in one of three mtDNA-encoded OXPHOS complex I genes: G11778A in the *ND4* gene, T14484C in *ND6,* and G3460A in *ND1* [11, 12]. These mtDNA mutations lead to decreased complex I enzyme rates and lowered ATP production [13] which are hypothesized to sensitize the RGCs to apoptosis. Patients have normal vision until the second or third decades, when a rapid loss of central vision occurs in one eye followed by the second eye within days to months. Visual loss progresses to 20/200 or worse, with visual field testing revealing central or centrocecal scotomas [9]. Axonal loss in the papillomacular bundle results in temporal atrophy of the optic nerve head. Maternal inheritance provides a strong diagnostic clue, but penetrance of LHON is variable within kindreds, and males are overrepresented with around 90 % of affected individuals being male. X-linked loci were postulated to explain the higher male penetrance, but major efforts since the 1990s have failed to identify strong or consistent candidate genes [14, 15] and the male bias remains unexplained.

A second major mitochondrial optic neuropathy is the Mendelian disease autosomal dominant optic atrophy (ADOA). The most common gene mutated in ADOA is *OPA1*, a dynein-related GTP-ase of the mitochondrial inner membrane that directs fusion of this membrane. ADOA results from haploinsufficiency of OPA1 protein, but how this causes specific loss of RGCs remains unknown. Disrupted inner membrane cristae structure is likely to impact on OXPHOS due to either inadequate mtDNA transcription or lack of inner membrane surface area for OXPHOS complex anchoring. We have reported decreased OXPHOS capacity in ADOA patients with *OPA1* mutations and relatively poor visual acuities, while related mutation carriers with normal vision appeared to have relatively preserved OXPHOS function [16]. This suggests that patients with preserved vision may harbor genetic variants that allow some compensation of OXPHOS.

2.3.2 Syndromic mtDNA Diseases with Retinal Involvement

Several syndromic central nervous system diseases are also known to result from mtDNA mutations. While optic neuropathy is sometimes found in these disorders, a pigmentary retinopathy with loss of photoreceptors is also common. A feature of mtDNA diseases exemplified by the following disorders is that the mutations are usually present in *heteroplasmic* form, where there is a variable mixture of wild-type and mutant mtDNA in individual cells and tissues. This is in contrast to LHON which is usually associated with *homoplasmic* mtDNA mutations [4]. Heteroplasmy has interesting consequences for stem cell modelling of these mutations as discussed below (Sect. 2.5).

Neurogenic atrophy and retinitis pigmentosa syndrome (NARP) results from point mutations in the mtDNA ATPase-6 gene, commonly T8993G. Patients typically present with retinitis pigmentosa with or without optic neuropathy and can develop dystonia [17]. Mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) can result from many mtDNA point mutations, although the most common is the A3243G mutation in the $tRNA^{Leu}$ gene. MELAS patients present with stroke-like episodes that lead to frequent retrochiasmal visual loss, but often also have pigmentary retinopathy without optic atrophy [18]. The Kearns–Sayre syndrome is the severest form of chronic progressive external ophthalmoplegia grouping (KSS/CPEO), resulting from heteroplasmic mtDNA deletions, where the presenting feature is usually ptosis and ophthalmoplegia. KSS patients also often develop a pigmentary retinopathy together with cardiac conduction defects and severe neurological signs including ataxia [19].

2.3.3 Mendelian Mitochondrial Syndromes with Retinal Involvement

Several nuclear gene mitochondrial disorders have optic neuropathy as part of a multisystem disease. These are considered briefly here since they result from mutations in mitochondrial proteins that indirectly interfere with OXPHOS, and so add further insights into energetic failure and retinal pathology.

Friedreich's Ataxia is caused by a GAA trinucleotide repeat expansion in the *frataxin* gene. Frataxin is a mitochondrial protein involved in iron-sulphur (Fe-S) cluster assembly, although the precise function of the protein remains elusive. Lower frataxin levels result in cellular iron dyshomeostasis with increased free iron in mitochondria suggesting disruption of normal iron incorporation into redox centres of proteins. A complex multisystem clinical picture results with many features of mitochondrial diseases, although ataxia and cardiac defects are the most debilitating [20]. Optic atrophy is a common feature; the pattern of RGC loss in Friedreich's ataxia being more diffuse than that seen in LHON and ADOA, not preferentially involving the papillomacular bundle but involving the optic radiations [11].

Because the OXPHOS pathway relies on the redox ability of iron, via multiple Fe-S clusters, to perform electron transfer and thus energy transduction, it is likely that OXPHOS dysfunction is important in pathogenesis.

Mohr–Tranebjaerg syndrome is an X-linked recessive disease characterized by deafness, dystonia, and optic atrophy. It is caused by mutation of the gene *TIMM8A* [21]. TIMM8A is one of several proteins forming the translocase of the inner membrane (TIM) complex, which together with the translocase of the outer membrane (TOM) forms the machinery of mitochondrial protein import [22]. Perturbation of mitochondrial protein import is likely to impact on the OXPHOS pathway, although mitochondrial studies in patients have not yet been reported. RGC loss appears to be similar to that reported for Friedreich's ataxia, with diffuse involvement of the optic radiations.

Hereditary spastic paraplegia is a disease grouping that results from mutations in several nuclear genes. It is characterized by progressive spasticity of the lower limbs, frequently complicated by the presence of optic atrophy. One variant is caused by mutations in the *SPG7* gene coding for paraplegin, an AAA-type metalloprotease of the mitochondrial inner membrane. Mutation of paraplegin may result in impairment of OXPHOS complex I, which if confirmed would provide a pathogenetic link to LHON [23].

Charcot–Marie–Tooth (CMT) disease subtype CMT2A has been associated with mutations in the mitochondrial fusion protein mitofusin-2 (Mfn2) [24]. CMT is a relatively common inherited peripheral neuropathy; the variant CMT2A also displays an optic neuropathy that develops after the neuropathy. A rapidly progressive blindness with bilateral central scotomas is evident on fundus examination, reminiscent of LHON [11, 12]. The pattern of RGC loss is also similar to LHON with preferential involvement of the papillomacular bundle [24]. Mfn2 is an outer mitochondrial membrane GTP-ase similar in structure to OPA1. OXPHOS studies have been limited and inconclusive.

Table 2.1 summarizes these mtDNA-linked and nuclear gene mitochondrial disorders with retinal involvement.

2.3.4 Glaucoma

The most common optic nerve disease is primary open angle glaucoma (POAG). It is an age-related neurodegenerative disease characterized by the accelerated death of RGCs leading to progressive visual field loss; it affects over 10 % of people aged over 80 years [25]. Apart from age the major risk factor is increased intraocular pressure, but up to 40 % of patients do not present with eye pressures above population means, indicating that glaucoma is a complex disease and age-related mitochondrial failure has been hypothesized to play a role [26]. We have found a partial complex I-driven respiration defect in glaucoma patient peripheral cells [27]. POAG is likely a multifactorial disease and further analysis of OXPHOS function is warranted and may uncover a subgrouping of patients with primary mitochondrial impairment.

Table 2.1 mtDNA-linked and mitochondrial nuclear gene disorders with retinal pathology

Disease	Gene(s)	Pathway	Retinal phenotype
LHON	mtDNA complex I subunit genes	OXPHOS	Optic neuropathy
Neurogenic atrophy and retinitis pigmentosa syndrome (NARP)	mtDNA ATPase-6 gene, commonly T8993G	OXPHOS	Retinitis pigmentosa with or without optic neuropathy, sometimes dystonia
Mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS)	mtDNA A3243G $tRNA^{Leu}$ gene (usually), complex I subunits (sometimes)	OXPHOS	Stroke-like episodes, pigmentary retinopathy
Maternally inherited diabetes and deafness (MIDD)	mtDNA A3243G mutation in the $tRNA^{Leu}$ gene	OXPHOS	Sensorineural deafness, retinal abnormalities, and diabetes
ADOA	*OPA1*	Mitochondrial fission/fusion, OXPHOS	Optic neuropathy
Friedreich ataxia	GAA trinucleotide repeat expansion in the *frataxin* gene	Mitochondrial iron-sulfur proteins including OXPHOS	Optic neuropathy
Mohr-Tranebjaerg syndrome (MTS)	*DDP1/TIMM8A*	Mitochondrial protein import, secondary OXPHOS defects	Optic neuropathy and retinopathy
Charcot–Marie–Tooth disease subtype CMT2A	*MFN2*	Mitochondrial outer membrane fusion defects	Optic neuropathy
Hereditary spastic paraplegia	For example *SPG7* (paraplegin)	OXPHOS complex I	Pathology not reported

The pattern of RGC loss in glaucoma differs to that seen in LHON and ADOA. In the latter diseases the small fibers of the papillomacular bundle are preferentially affected, resulting in a temporal atrophy and central vision loss. In POAG peripapillary atrophy classically leads to an arcuate, peripheral vision loss. As noted above for the syndromic mitochondrial optic neuropathies, different patterns of optic nerve, retinal, and wider visual system pathology can result from mitochondrial dysfunction, such that the pathological pattern of fibre loss cannot exclude a mitochondrial etiology.

2.3.5 Age-Related Macular Degeneration

Age-related macular degeneration (AMD) is a late onset neurodegenerative disease divided into two major forms; ‘wet AMD’ caused by local rupturing of blood vessels, and the far more common ‘dry AMD’ which is associated with the build-up of

protein deposits called drusen in the macula. Dry AMD is driven by a disruption in the close interrelationship of the retinal pigment epithelium (RPE), the choroid, and the dependent photoreceptors [28]. Several genetic risk factors have been found, including complement factor pathway protein genes and mtDNA haplotypes [29] but pathogenesis remains poorly understood.

The RPE forms the retina-brain barrier and is very metabolically active; it is richly endowed with mitochondria [7]. Oxidative stress is increased in this tissue due to the high local oxygen concentrations from proximity to the choroid and the continuous photoreceptor outer segment phagocytosis by the RPE that leads to accumulation of the phototoxin *N*-retinyl-*N*-retinylidene ethanolamine. It is tempting to speculate that RPE/photoreceptor pathology as seen in some mtDNA diseases and AMD may be related to oxidative stress, while optic atrophy is a common consequence of energetic deficiency.

2.4 Mitochondrial Energetics in Stem Cells: The 'Metabolic Switch'

Recognition that stem cells and their differentiated progeny have distinct metabolic profiles has increased interest in mitochondrial control of metabolism in stem cell research. Quiescent or dividing stem cells have been found to use glycolysis for ATP production, and mitochondrial ATP production is shut down [30, 31]. 'Stemness' may in itself be an evolutionary protection mechanism to minimize oxidative mutagenesis of the organellar genome [32]. mtDNA has long been known to undergo higher levels of mutation in somatic cells compared to nuclear genes [33], possibly as a result of the close proximity of the genome to the OXPHOS machinery which is a constitutive source of superoxide. Stem cells with quiescent OXPHOS may be one means of preserving the mtDNA until it is most needed in metabolically demanding differentiated cells such as neurons.

The reprogramming of somatic cells to pluripotency (induced pluripotent stem cells or iPSCs) also involves downregulation of oxidative metabolism and upregulation of glycolysis [34]. Other major metabolic pathways found to undergo remodelling include upregulated purine metabolism and macromolecule catabolism, with downregulation of amino acid metabolism and nucleotide biosynthesis [34].

Conversely the onset of differentiation has been shown to be marked by upregulation of OXPHOS [30, 35]. Differentiation is accompanied by an increase in mtDNA replication [36], increased expression of key mitochondrial biogenesis regulators such as PGC-1α, mitochondrial volume, and oxygen consumption [37]. Differentiated cells have varying dependence on mitochondrial oxidative ATP production, with some cell types not relying heavily on OXPHOS. The cell type in model systems is therefore an important variable when examining mitochondrial influences. For example fibroblasts are not highly oxidative while neurons and cardiomyocytes are OXPHOS-dependent, reflecting the predilection for pathology in the latter tissues in mitochondrial diseases [3].

2.5 Stem Cell Modelling of mtDNA Mutations

Of the mtDNA-linked human retinal diseases, only the MELAS A3423G $tRNA^{leu}$ mutation has been studied in stem cell models to date. Fujikura et al. [38] reported iPSCs derived from fibroblasts of patients carrying the mutation in heteroplasmic form. They found that clonal iPSC lines varied greatly in the levels of mutation, with some clones segregating to wild type and others maintaining high mutant levels. This has clear implications for potential stem cell therapy of mtDNA disease and demonstrated the feasibility of eliminating the pathogenic mutation in isogenic cell lines from patients. The phenomenon of in vitro segregation of this mutation was replicated in another study that found a bimodal distribution of mutation load in patient-derived iPSCs [39]. They also found an intriguing potential mechanism for complex I-linked neuron-specific pathogenesis, showing that complex I in high-mutant iPSC-derived neurons was largely sequestered in perinuclear autophagosomes, while other OXPHOS complexes showed normal mitochondrial distribution. This suggests a neuron-specific derangement of complex I assembly consequent to the $tRNA^{leu}$ mutation and demonstrates the power of stem cell modelling of mtDNA disease.

Cybrid modelling of mtDNA disease presents unique experimental opportunities compared with nuclear gene mutation models. Cybrids ('cyoplasmic hybrid') involve fusion of enucleated cell fragments or cytoplasts with cells that have been depleted of mtDNA [40]. The technique has been used extensively with cultured cell lines to show the segregation of OXPHOS phenotypes with mtDNA mutations causing human diseases including the MELAS A3423G $tRNA^{leu}$ mutation [41], the NARP T8993G mutation [42], and the three primary LHON mutations [13].

As first demonstrated by Wallace's group [43] and followed by the creation of a 'xenomitochondrial' mouse using the same approach [44], the transfer of mtDNA from a mouse donor cell line of choice into mouse embryonic stem cells (ESCs) can be achieved by first depleting the ESCs of endogenous mtDNA. This is performed by treatment of the cells with the toxic (but non-mutagenic) dye rhodamine 6G and 'rescue' of treated cells by fusion with cytoplasts from the donor cell [45]. Remarkably, this drastic series of manipulations does not appear to result in epigenetic or other perturbations to the resulting cybrid ESC which retains pluripotent potential as evidenced by the normal development of the xenomitochondrial mice produced by blastocyst injection of these cells [44, 46]. The cell-specificity of OXPHOS defects has been reinforced by other in vitro mouse ESC cybrid studies examining effects of mtDNA mutations identified in mouse cell lines, also using the rhodamine 6G technique. These elegant studies found that mtDNA mutations causing bioenergetic defects resulted in impaired differentiation to neurons and decreased post-synaptic events [47] and increased oxidative stress in ESC-derived neurons with severe OXPHOS complex I defects [48].

Cybrid production has also been demonstrated using the rhodamine 6G method in primary human fibroblasts [49]. This opens the possibility of transmitochondrial modelling in iPSCs derived from fibroblast cybrids. This has not yet been reported, nor has direct cybrid transfer in either human ESC lines or iPSC lines.

2.6 Conclusions

Retinal neurons, especially RGCs, are among the most susceptible neurons to mitochondrial energetic impairment. It remains unknown why some defects in the OXPHOS pathway lead to loss of RGCs, while other defects lead to photoreceptor loss in the absence of optic neuropathy. One possibility is that OXPHOS defects can result in either ATP deficiency or oxidative stress to varying degrees. The RPE which forms the retina-brain barrier is highly metabolic. Oxidative stress in this tissue is likely to be very high. It is possible that the point mutations responsible for MELAS and NARP, and the mtDNA deletions associated with KSS/CPEO result in heightened oxidative stress compared with the LHON mutations. Histopathology in cases of mtDNA-linked pigmentary retinopathy supports a secondary disruption of photoreceptors consequent to RPE failure [9].

Both LHON and ADOA can occur in more 'complex' forms with variable central nervous system involvement. Extra-ocular features of 'LHON plus' syndromes include dystonia, ataxia, severe progressive encephalopathy. The mtDNA mutations in these cases are usually in complex I genes and different to the 'primary' LHON mutations. Where investigated these mutations have more severe defects in OXPHOS [50–52]. The clinical and biochemical evidence therefore supports the concept that mild OXPHOS defects, especially in complex I genes, result in preferential RGC loss. More severe OXPHOS defects result in wider CNS involvement, typically affecting the brainstem, basal ganglia, or cerebellum. The common involvement of sensorineural deafness and peripheral neuropathy in such patients suggests that neurons with long axons are more vulnerable to mitochondrial dysfunction.

Stem cell modelling of these different mutations presents an ideal experimental system to test this hypothesis, with direct implications for therapeutic targets. Effects on different neuronal types can be tested in vitro, along with therapeutics that may be directed to augmenting oxidative stress defenses, mitochondrial biogenesis, or other 'energetic' therapies.

Just as nuclear gene changes have been found in some iPSC-derived cells compared with parental somatic cells, mtDNA mutations are also at risk of becoming fixed in such cell lines. Prigione et al. [53] investigated this directly by massively parallel pyrosequencing of mtDNA from iPSCs and found both heteroplasmic and homoplasmic mtDNA mutations not present in parental somatic cells. The important implication is that for stem cell-based therapeutic approaches in the future it will be necessary to verify the full mtDNA sequence of iPSCs used.

For mtDNA disease mutation research and therapy development stem cell cybrid techniques offer a unique opportunity for 'correction' of mtDNA mutations in isogenic iPSCs. Again, the donor mtDNA needs to be carefully matched and ideally from a maternal lineage relative that does not carry the mutation even in low level heteroplasmic form. This is because a remarkable study found that mixture of two non-disease causing mtDNA haplotypes in mice resulted in abnormalities of development that were absent when mice had either mtDNA haplotlype in homoplasmic form [54]. This underscores the adaptation between the

mitochondrial genotype and nuclear gene expression, likely by epigenetic feedback mechanisms still poorly understood [6, 55], that is an underexplored aspect of the basis of eukaryotic complexity.

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Chapter 3
The Regenerative Potential of the Vertebrate Retina: Lessons from the Zebrafish

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Contents

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A. Pébay (ed.), *Regenerative Biology of the Eye*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4939-0787-8_3,

Abbreviations

ADP	Adenosine diphosphate
Ascl1a	Achaete-scute complex like 1a
Atoh7	Atonal homolog 7
ATP	Adenosine triphosphate
bHLH	Basic helix loop helix
Bmp	Bone morphogenetic protein
Brn3b	Brain-specific homeobox 3b
CGZ	Circumferential germinal zone
Chx10	Ceh-10 homeodomain containing homolog
CMZ	Ciliary margin zone
CNTF	Ciliary neurotrophic factor
Crx	Cone rod homeobox
Dkk1b	Dickkopf 1b
Dll1	Delta-like 1
Dpi	Days post-injury
Drgal1-L2	β-Galactoside-binding protein galectin 1-like 2
ERG	Electroretinogram
Fgf8	Fibroblast growth factor 8
FoxN4	Forkhead box N4
Fzd2	Frizzled 2
Gap43	Growth-associated protein 43
GCL	Ganglion cell layer
GFAP	Glial fibrillary acidic protein
GSK-3β	Glycogen synthase kinase-3β
HB-EGF	Heparin-binding epidermal like growth factor
Hes5	Hairy and enhancer of split 5
Hpi	Hours post-injury
Hspd1	Heat shock 60-kDa protein 1
Id2a	Inhibitory of differentiation 2
IgF	Insulin growth factor
IKNM	Interkinetic nuclear migration
INL	Inner nuclear layer
Insm1a	Insulinoma-associated 1a
MAPK	Mitogen-activated protein kinase
Mcm	Minichromosome maintenance protein
Mps1	Monopolar spindle 1
Ngn1	Neurogenin 1
NMDA	*N*-methyl-D-aspartate
Oct4	Octamer-binding transcription factor 4
Olig2	Oligodendrocyte transcription factor 2
ONL	Outer nuclear layer
Pax6	Paired box 6

PCNA	Proliferating cell nuclear antigen
PDGFA	Platelet-derived growth factor A
Rac1	Ras-related C3 botulinum toxin substrate 1
Shh/Hh	Sonic hedgehog/Hedgehog
Six3b	Sine-oculis homeobox homolog 3b
Sox2	Sex determining region Y-box 2
Stat3	Signal transducer and activator of transcription 3
TGFβ	Transforming growth factor beta
Tgif1	Transforming growth interacting factor
TNFα	Tumour necrosis factor alpha
Trb	Thyroid hormone receptor β
Tuba1a/α1T	α_1-Tubulin
UAS	Upstream activating sequence
Vsx1/Vsx2	Visual homeobox transcription factors 1 and 2

3.1 The Zebrafish Model for Studying Retinal Regeneration

Here, we highlight the advantages of the zebrafish model for this research field. Firstly, we describe the vertebrate species conservation in terms of cell types, retinal organisation and developmental mechanisms. We then summarise aspects of zebrafish adult neurogenesis and its regenerative capacity, focussing on what we have learned about the regenerative response driven by the endogenous Müller glia cells.

3.1.1 Regenerative Capacity of Zebrafish

A distinct capacity for different organs to regenerate exists across different animal phyla, with generally lower species showing more robust and complete regeneration [1, 2]. After retinal injury, all vertebrates show a differential response, with amphibians and fish displaying the best regenerative response, in terms of replacing lost or injured cells, followed by birds and to a lesser extent by mammals [3]. Species differences in how many cells are activated to contribute and how many cells are regenerated in different injuries, and the ability to regenerate some or all of the retinal cell types may be due to intrinsic genetic differences of regenerating cells, or due to extrinsic signalling differences in the damaged tissue. Thus, understanding and comparing highly regenerative models with vertebrates where only a few cells regenerate allows us to differentiate between intrinsic mechanisms we cannot easily change in vivo, and extrinsic signalling pathways that we can target and improve.

Zebrafish have retained highly pro-regenerative responses to injury of many different body organs, such as fin, heart, spinal cord, brain and retina [4]. Its amenability for developmental and regenerative studies make it a great model for

retinal regeneration. Additionally, the conservation of retinal structure and development between zebrafish and other vertebrates suggests that knowledge gained in this model will be relevant across vertebrate species.

3.1.2 Zebrafish Retinal Organisation

The anatomical structure, cell types and organisation of the neural retina are conserved across all vertebrates. All vertebrate retinas are composed of six main types of retinal cells: Three excitatory neurons (photoreceptors, bipolar and ganglion cells), two inhibitory neurons (amacrine and horizontal cells) and Müller glia cells. These cell types are arranged in a conserved ordered retinal lamination, consisting of three nuclear layers (outer nuclear layer—ONL, inner nuclear layer—INL and ganglion cell layer—GCL), separated by two plexiform layers housing processes and synaptic connections. Each neuron type can be further subdivided into subtypes based on their location, morphology, gene expression and function [5–10]. Relevant particularly for human retinal regenerative studies, zebrafish are highly visual vertebrates with a cone-photoreceptor-dominated retina similar to the human fovea, which is in contrast to some of the nocturnal mammalian models (e.g. rodents). Thus, it is a particularly suitable vertebrate model to study specific photoreceptor loss and subsequent regeneration.

3.1.3 Zebrafish Retinal Development

Because regeneration involves the generation of the same cell types that are produced during development, the vast knowledge we have gained about retinal development from the zebrafish model represents an advantage in studying mechanisms of regeneration. The zebrafish has become a leading developmental vertebrate model system, due to a number of advantages including large clutch sizes, amenability to molecular manipulations, rapid ex vivo development and transparency, allowing for rapid generation of transgenic lines, in vivo imaging, gene manipulations, forward and reverse genetics.

Developmental mechanisms by which the retina is constructed are highly conserved, with comparable genetic control and hallmarks evident in zebrafish as in other vertebrates, making studies of the zebrafish relevant to understanding mammalian development. In all vertebrates retinal neurons are born in roughly the same histogenic birth order, starting with retinal ganglion cells followed by interneurons such as amacrine cells and then photoreceptors, with bipolar and Müller glia cells generated last [11–17]. This stereotypical progression of cell fate specification is due to the highly choreographed spatio-temporal expression of specific genes within developing multipotent progenitors. These include those that control cell cycle progression as well as those that drive determination of specific neural fates. Many of these genes encode for basic helix loop helix (bHLH) and homeobox transcription factors [18–21], which have conserved functional in zebrafish, mice and humans [22–31].

Armed with a wealth of data and growing understanding of how retinal cells are generated during embryonic development, we can now use this model and its regenerative capacity to understand how neurons are regenerated in the adult retina.

3.1.4 Therapeutic Approaches for Improved Regeneration

Different therapeutic approaches to treat retinal diseases or improve regeneration are being developed to find solutions to retain or restore visual function. No doubt, treatment strategies will vary as widely as retinal diseases or injuries. The main therapeutic approaches currently being pursued include increasing neuroprotection via extrinsic factors or from endogenous support cells, discovering suitable sources for cell replacement by driving differentiation ex vivo and transplanting exogenous cells or activating endogenous cell sources, as well as other biotechnology approaches including retinal implants currently already at clinical trial stages. Great progress has been made in the cellular transplantation field particularly in the replacement of photoreceptors [32–35]. Clinical trials delivering genes to treat genetic degenerative diseases are also underway [36]. With the advances of the stem cell field, new potential sources of replacement cells are being discovered and the potential of integrating these for use in regenerating different cell types continues to be pursued [37–40].

Contributions to our understanding of retinal regeneration driven endogenously in pro-regenerative vertebrates such as the zebrafish have significantly expanded in the past decade, from identifying neurogenesis in the adult central nervous system and a systematic search for stem cells, to the identification of endogenous cell sources for retinal regeneration [41]. Here, we summarise aspects of zebrafish regeneration, including a description of adult neurogenesis and different injury/regeneration models. The main focus will be on our current understanding of the Müller glia, the main endogenous cell source of regeneration, what we know about the Müller glia driven pro-regenerative response, which signalling pathways are involved, how this compares with normal development, and how some of these findings tie in with mammalian retinal regeneration.

3.2 Sources of Retinal Progenitor Cells in the Adult Zebrafish

3.2.1 Adult Neurogenesis

Despite the conservation of morphology and gene regulation between vertebrate species, the regenerative capacity and source of regeneration differ. In zebrafish, our understanding of adult neurogenesis in the central nervous system including cell sources and gene expression has grown significantly in recent years [42]. As an outpocketing of the central nervous system, the retina of non-mammalian species such as zebrafish, chicken and *Xenopus* is observed to have an extensive capacity of

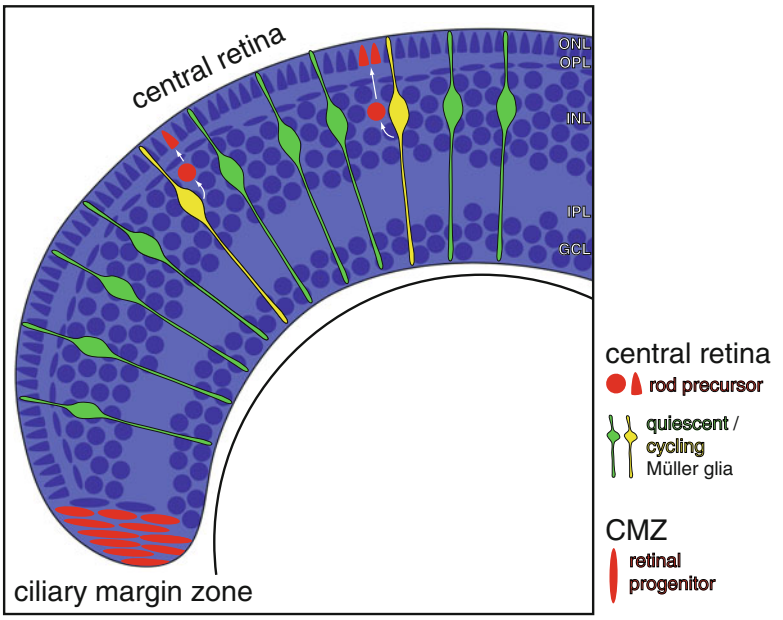

Fig. 3.1 Retinal progenitor compartments in adult fish. The growth and cellular addition in the uninjured adult fish arises from two main compartments. In the ciliary margin zone (CMZ), progenitors (*red oval* nuclei) continuously add new cells to the growing retina. In the central established region, some of the mature Müller glia are slowly cycling (*yellow* glia) to generate rod precursors (*red circular* nuclei), which migrate into the outer nuclear layer (ONL) to add rod photoreceptors (*red* photoreceptor). The majority of Müller glia in the adult retina remain quiescent (*green* glia). *ONL* outer nuclear layer, *OPL* outer plexiform layer, *INL* inner nuclear layer, *IPL* inner plexiform layer, *GCL* ganglion cell layer

postnatal growth and regeneration after injury [43, 44]. In contrast, cell proliferation in mammalian retina ceases after birth, although the eye and retina continue passive growth by stretching [45]. Although potential sources of retinal stem cells have been suggested, studies of active retinal regeneration in in vivo models are necessary to better understand and improve this process in mammals.

There are at least two retinal progenitor compartments in adult fish [43] (Fig. 3.1). The resident progenitor cells in the ciliary margin zone (CMZ) or circumferential germinal zone (CGZ) found in teleost, birds and amphibians are the major retinal stem cell source in uninjured, mature retinas. These cells recapitulate developmental gene expression to add cells continually to the retina throughout the lifetime of the organism [46–52]. An additional source of retinal progenitor cells in mammals and mature teleost retina are Müller glia, though not all glia in the differentiated retina are associated with retinal neurogenesis [3, 37, 40]. Müller cells are the major retinal glia cell type and the only one generated directly from retinal progenitors. Their processes span the entire retina radially, surrounding neuronal cell bodies, while their nuclei reside in the INL. Like other glia cells of the nervous system, Müller glia perform a wide range of functions, including recycling neurotransmitters, maintaining ion homeostasis and regulating neuronal survival and circuit formation in the retina [3, 53–57]. Müller glia with slow, but ongoing proliferative activity, generate clusters

of cells in the central differentiated retina. These migrate to the ONL to form rod precursors, which divide and differentiate into mature rod photoreceptors [44, 58–61] (Fig. 3.1). Actively dividing Müller glia associated with adult neurogenesis are scattered throughout the retina, with greater density at the CMZ [59, 62].

3.2.2 Adult Regeneration

Zebrafish can generate retinal cells upon retinal injury from different endogenous cells including those in the CMZ and rod precursors [63–65]. Resident Müller glia have become the focus of intensive research, as they are now believed to give rise to the majority of regenerating cells [60, 66, 67]. The pigment epithelium has also been identified as a source of retinal regeneration in other vertebrates, especially in amphibian [3]. Different injury models result in a regenerative response from different endogenous cells, arguably due to differences in the microenvironment. The extent to which the CMZ responds to retinal injury has not been studied in detail.

After injury, resident Müller glia are activated, dedifferentiate as they re-enter the cell cycle and generate multipotent retinal progenitors, which can replenish all retinal cell types ultimately leading to functional recovery [66, 68, 69]. As Müller glia are present in all vertebrates, understanding and comparing their intrinsic limits in the regenerative potential in different species will allow us to develop strategies to potentially stimulate a regenerative response in humans.

The regenerative response of Müller glia in higher vertebrates including birds and rodents occurs to a limited degree, both in quantity and types of neurons generated [3, 70]. In birds, Müller glia express markers of embryonic retinal progenitors and re-enter the cell cycle, but only for one division and only a small percentage of Müller glia progeny successfully differentiates into new retinal neurons [70–72]. In rodents, also only a small percentage of Müller glia re-enters the cell cycle after injury [73], though a more extensive injury can increase the number of proliferating Müller glia [74]. Isolation of mammalian Müller glia from injured retina showed limited self-renewal and stem cell-like characteristics in vitro, suggesting that they can be similarly activated [75, 76]. Even human Müller glia display a regenerative capacity to undergo indefinite proliferation and differentiation towards some retinal neuron fates at least in vitro [77, 78]. The number of cells proliferating and the number of cell divisions each cell undergoes remains one of the main limitations in mammals. Identification of their potential to initiate a proliferative response however suggests the potential for glial-driven mammalian regeneration and provides hope for this endogenous regenerative cell source.

3.3 Zebrafish Retinal Injury Models

Various injury and regeneration models have been established in zebrafish, which elicit robust regenerative responses from resident Müller glia (Fig. 3.2a). Different models target different types of neurons, differ in the extent of injury and mimic retinal disorders resulting from chemical, mechanical and genetic causes. Established

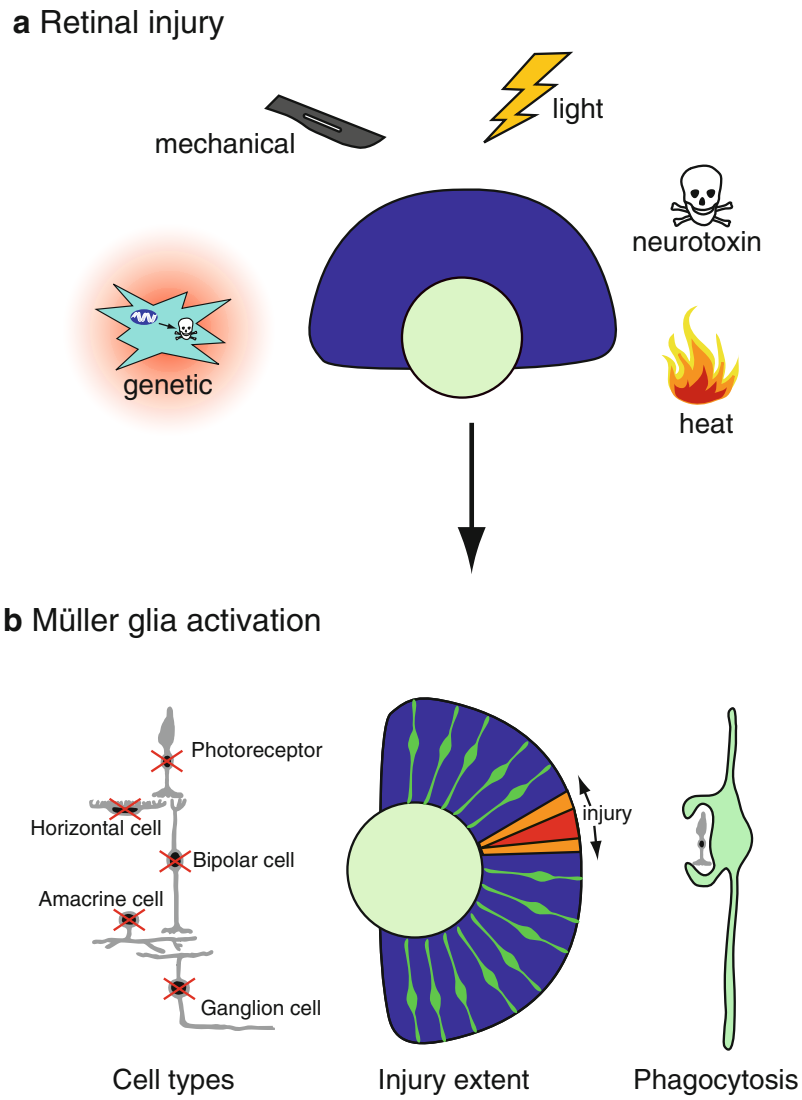

Fig. 3.2 Retinal regeneration initiation in zebrafish Müller glia. (**a**) The vast majority of the different injury models established in zebrafish (including mechanical, light- or heat-induced damage, genetic ablation or delivery of various neurotoxins) activate a robust retinal regenerative response from the resident Müller glia. (**b**) The activation of glia and the resulting regeneration differs and depends on injury-specific factors such as the retinal cell types affected (injured), the extent of the injury and the phagocytic function of mature glia

retinal injury models include: (1) Mechanical injuries such as surgical lesions [64, 79, 80], needle stab [81] or optic crush [66, 82]; (2) Constant or intense light [60, 83–85], heat [48] or laser lesioning [65, 67]; (3) Chemical lesioning using neurotoxins such as ouabain [63, 86, 87], kainic acid [88, 89], colchicines [90, 91], *N-methyl-d*-aspartate (NMDA) [91, 92] or *N*-methyl-*N*-nitrosourea [93]; and (4) Genetic approaches

coupled with chemical ablation, such as using a cell-specific promoter to drive Gal4 expression, which in turn activates upstream activating sequences (UAS) to restrict nitroreductase expression within these cells, subsequently causing cell death after metronidazole treatment [94–98]. Differences between these paradigms include the extent of injury and cell types involved [43, 99, 100]. It is unknown whether the same signalling mechanisms are activated in these injury models, although differences in the timing of the regenerative response have been observed.

3.4 Müller Glia Activation

3.4.1 Cell Death Extent of Distinct Retinal Neuron Types

Müller glia activation depends on various factors, including neuron cell type affected (Fig. 3.2b). As many human degenerative diseases such as retinitis pigmentosa or age-related macular degeneration affect photoreceptors, early studies focused on light lesioning and genetic ablation paradigms to cause specific loss of these neurons. Activation of the regenerative response depends on the type of photoreceptor damaged and extent of damage. For example, low insult of rod photoreceptors alone primarily activate rod precursor-driven regeneration, while a massive insult to the retina resulting in major rod and/or cone photoreceptors ablation activates Müller glial-derived regenerative response [60, 69, 85, 97, 101–103]. In addition, acute, but not chronic, rod photoreceptor death induces widespread Müller glia activation suggesting that insufficient numbers of rod progenitors in the acute model may trigger subsequent glial involvement [97]. Recent findings causing damage to inner retinal neurons result in robust glial activation with minimal photoreceptor damage [66, 68, 86, 87]. Thus, while varied, a glial-driven regenerative response occurs in most models of retinal damage.

3.4.2 Phagocytosis

The phagocytic activity of Müller glia has been described for a long time, including early in vivo evidence from the rabbit retina [104–107], and in vitro evidence in goldfish [108] and humans [109].

After retinal injury, activated Müller glia phagocytose dying cells [110, 111] as they do during development [112]. The action of engulfing apoptotic debris is in itself a necessary signal to initiate proliferation, as demonstrated by experiments blocking phagocytosis using intravitreal injections of *O*-phospho-l-serine. After light lesioning, retinas, where phagocytosis is inhibited, show a reduced number of cells expressing the proliferating cell nuclear antigen (PCNA), leading to reduced number of regenerated photoreceptor cells [113]. Whether phagocytotic activity is equally important for Müller glia activation in other vertebrates is less understood.

3.5 Müller Glia Dedifferentiation, Cell Cycle Entry and Progenitor Reacquisition

Müller glia activation, while certainly driven by extrinsic signalling factors, also depends on the intrinsic capacity to sense or respond to these signals. Müller glia monitor the overall health of the retina and are primed to respond to factors that signal cell death or damage. In zebrafish, after injury, more Müller glia than those associated with the normal basal adult rate of proliferation re-enter the cell cycle.

3.5.1 Müller Glia Markers

Though similar to progenitors in many aspects, the maturation of Müller glia involves genetic, morphological and electrophysiological changes, and thus regeneration requires glial dedifferentiation [54]. It is still unclear whether all Müller glia have an equal capacity to be involved in regeneration. Within the responsive area, some Müller glia continue to retain mature glial morphology and expression of the glial fibrillary acidic protein (GFAP) glia marker, whereas a subpopulation start to lose their glial morphology and differentiation markers and start re-expressing cell cycle marker (e.g. PCNA) and activate signalling pathways involved in proliferation [69, 114]. Gradients of signalling pathway interaction may contribute to this differential response of glia [115]. Evidence for differential glial response can also be found in chick, older Müller glia in central retina stop responding to toxin-induced injury [70]. Because not all Müller glia in the teleost participate in retinal regeneration after mechanical injury [62, 66], there may be inherent heterogeneity. Some studies have found no molecular differences to identify which glia proliferate and which remain quiescent, although molecular differences do appear in these differentially responding Müller glia after injury in zebrafish and chick [116–118]. Whether this differential response is due to stochastic activation of a homogeneous population followed by differential gene expression or whether these glia are inherently different must be investigated in more detail. In mouse, variation in the expression of various retinal progenitor and glial genes does exist, suggestive of inherent heterogeneity [119].

While the type of injury does not influence the temporal order of the resulting proliferative response in Müller glia, different lesioning methods differentially influence the expression of various glial markers in various vertebrates. The Müller-specific marker glutamine synthetase can be turned off after injury such as that induced by intense light injury [53, 69], upregulated in the case of hepatic retinopathy models, or remain unaltered such as in diabetic retinopathy or optic crush models [120, 121]. Similarly the radial glial marker GFAP, which is upregulated during stress and during pathological processes in mammalian retina [65, 67], shows differential responses. After toxin-induced injury in the chick retina, Müller glia that have decreased GFAP staining re-enter the cell cycle [70]. In zebrafish, GFAP levels have been observed to be upregulated after injury, including after heat probe injury of the sclera and in response to light lesioning [48, 103], though dedifferentiating regenerating glia specifically downregulate GFAP after light or ouabain lesioning [69, 86]. This differential regulation of glial markers may correspond to distinct phases of the glial response or

be a reflection of response heterogeneity, with glia activating alternate responses. Other genes associated with mature glia function are downregulated during the dedifferentiation phase including those that are associated with ion homeostasis [122]. The variation observed in Müller glia marker expression after injury suggests differences in the initial response, which may activate distinct repair mechanisms. Moreover, the difference in expression levels of these markers could be due to the extent and/or type of damaged caused and cell types involved.

3.5.2 Gliosis Versus Neurogenesis

As observed in other injury models, activated glia can respond in very different ways. Following damage in the mammalian retina, Müller glia generate both a protective and a degenerative response [73]. Depending on the type and severity of the injury, Müller glia undergo morphological, biochemical and physiological changes including reactive gliosis, characterised either by hypertrophy with infrequent or no Müller glia proliferation [123–126] or upregulated proliferation to form a glial scar, together with a decrease in protein expression associated with normal retinal physiology [56, 127–129]. Reactive gliosis involves upregulation of neuroprotective intermediate filaments such as Nestin, Vimentin and GFAP that affect Müller glia morphology and thus function [129, 130]. Reactive gliosis may facilitate revascularization, form physical or diffusion barriers and promote the secretion of neurotrophic factors. However, acute inflammation followed by reactive gliosis also leads to glial scar formation, which inhibits survival, neurite growth and circuit integration of neurons [131].

There are similarities and differences in the response of Müller glia in zebrafish, although it is unclear whether differences are due to intrinsic differences or driven by environmental signals at the injury. Zebrafish Müller glia also undergo characteristic changes with some features similar to reactive gliosis in mammals, such as initial GFAP expression. However, Müller glia in zebrafish re-enter the cell cycle, dedifferentiate and migrate to the apical surface (characteristics of immature Müller glia) without forming a gliotic scar [44]. Acute inflammation itself in the zebrafish appears to be required and sufficient for increasing neuron progenitor proliferation and neurogenesis by activating signalling molecules that are pro-regenerative after brain injury [131]. Thus, this initial response of Müller glia in mammals may not be harmful, nor mutually exclusive with subsequent cellular regeneration.

3.5.3 Cell Cycle Re-entry and Proliferation

The early response of Müller glia to retinal injury involves cell cycle entry, and progenitor and proliferation maintenance. Proliferation itself is a crucial step, as blockage of glial proliferation results in a failure of retinal neuron regeneration [69]. A proliferative response of glia cells in other vertebrates including birds and rodents does occur to a limited degree [70–73].

After an initial phase characterised by the upregulation of protein synthesis and cell metabolism, Müller glia proliferate, with the proliferation peak being surprisingly similar across different injury models even though different cell types are damaged. For example, both light-induced and ouabain-induced damage results in glial activation and increased expression of PCNA within 24 h post-injury (hpi) [69, 85, 86, 132]. Dedifferentiation of Müller glia (reduction in GFAP labelling) starts at 3 dpi and overlaps with the proliferative phase, which peaks at 5 dpi, resulting in a gradual decline of GFAP/PCNA double-labelled Müller cells [86]. PCNA-labelled Müller-derived (olig2:eGFP positive) cells form clusters around the GFAP-positive Müller glia at 3 dpi and re-acquire the ability to migrate across retinal layers [86].

Similar to the early stages of development, this early phase involving cellular expansion must be carefully controlled and balanced to promote sufficient proliferation to generate the correct number of new neurons, but also stop proliferating to give rise to differentiating cells [87, 133]. Here, we summarise the main factors involved in balancing this phase.

Microarray expression studies of light damaged retinas have revealed differential expression changes in a whole cohort of genes involved in activating DNA synthesis, general cell function regulation, cell growth such as upregulation of minichromosome maintenance protein family (mcm 3,4,5 and 7), PCNA, Cyclin d1, Cyclin B1, signal transducer and activator of transcription 3 (Stat3), growth-associated protein 43 (gap43), α1-tubulin (α1T or tuba1a), platelet-derived growth factor (PDGFA) and downregulation of cullin 3 and cullin 5 ubiquitin ligases [103, 122, 134–136].

The temporal expression patterns of different factors [4] indicate at which stage these genes are most likely to act. Gorsuch and Hyde [115] and Lenkowski et al. [137] recently summarised the cross-regulation and interactions of the main identified molecular players involved in co-ordinating this glial dedifferentiation, proliferation and acquisition of progenitor like phenotype (Fig. 3.3).

The tumour necrosis factor alpha (TNFα) is an important extrinsic cue released by dying retinal neurons and together with Achaete-scute complex like 1a (Ascl1a) and Stat3 is required for the proliferative response [115, 138, 139] (Fig. 3.3b). However, Stat3 is also turned on in Müller glia that do not turn on Ascl1a and that do not proliferate [118]. Transforming growth factor beta (TGFβ) signalling acting through Activin plays an important role during the proliferative and differentiation stage [137]. Transforming growth interacting factor (Tgif1) and Sine-oculis homeobox homolog 3b (Six3b) are upregulated early after injury and repress TGFβ signalling via Smad2, 3. Ascl1a acts as the master regulator of the retinal regenerative response and may thus represent one of the avenues to improve mammalian regeneration. Ascl1 interacts in complex signalling loops with multiple signalling pathways. The downstream activation of Lin28 and suppression of let7 microRNA drives many of the regeneration-associated processes, including proliferation and re-aquisition of progenitor like multipotency [140]. Even though mammalian glia are activated to respond to injury, important differences exist, such as the lack of Ascl1 upregulation [2]. Pollak et al. [141] recently showed that Ascl1 itself is sufficient to reprogram mature mouse Müller glia into neurogenic retinal progenitors (downregulating glia markers, upregulating progenitor markers and generating specific retinal neurons) [141], providing exciting evidence that mammalian glia have a similar capacity to respond similarly, if the correct pathways are activated.

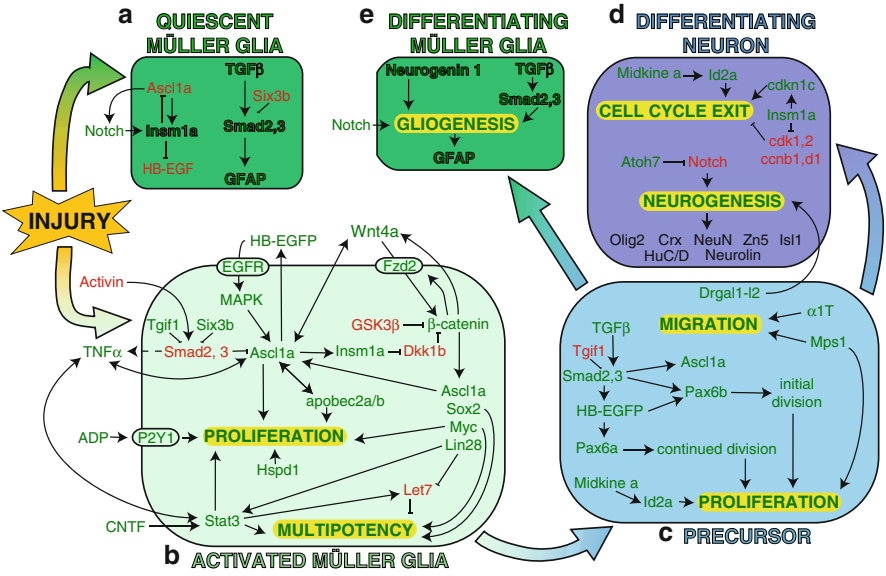

Fig. 3.3 Schematic showing interactions between some of the main signalling pathways during retinal regeneration. (**a**) Upon retinal injury, some Müller glia within the regenerative zone and further away remain quiescent. (**b**) Other Müller glia respond to various extrinsic signals, including factors released by dying cells to undergo a regenerative response. In these activated Müller glia, many different signalling pathways interact to cause re-entry into the cell cycle (proliferation) and upregulation of progenitor like multipotency genes, which have been detected by various microarray type studies in recent years. These glia also dedifferentiate at this time by downregulating mature glia markers. Central to the network identified so far is the early transcription factor Ascl1a, which forms various feedback loops with signalling cascades such as canonical Wnt/β--catenin, TNFα, HB-EGFP, Stat3 to drive this response. Many extrinsic factors including TGFβ and ADP are also influential in driving this response. (**c**) Clonal expansion occurs in precursors that maintain proliferation by upregulating signalling pathways including Pax6a, b, TGFβ and Midkine a. At this stage of regeneration, other factors are upregulated, such as Mps1, N-cadherin and α1-tubulin to influence the migration of precursors through the retinal layers. (**d**, **e**) A subsequent change in signalling drives cell cycle exit and simultaneously drives either neurogenesis (**d**) or gliogenesis (**e**) to replace retinal neurons and Müller glia. Factors upregulated at the various stages are indicated in *green*, those downregulated are indicated in *red*. *Arrows* show cross-regulation, which may be direct or indirect. See main text for abbreviations

3.5.4 Progenitor Markers

Müller glia re-activate many progenitor markers including octamer-binding transcription factor 4 (Oct4), Nanog, Myc and sex-determining region Y-box 2 (Sox2) [140]. Ongoing efforts are characterising to what extent Müller glia-derived proliferating cells can be considered true stem cells or equivalent to developmental progenitors.

During retinal development Müller glia are the last cell type generated. Müller glia retain similarities with these early retinal progenitors, although important

differences are observed. These include protein expression that is important for the role mature glia carry out in the normal adult retina. cDNA microarray studies have revealed a striking similarity of retinal progenitors with Müller glia [119, 142]. Müller glia and proliferative cells originating from dedifferentiated glia share stem cell markers found during normal development and in progenitor cells found in the CMZ of adult zebrafish, including Visual homeobox transcription factors 1 and 2 (Vsx1, Vsx2), Notch1, Notch3, N-cadherin, Paired box 6 (Pax6), α1T, Atonal homolog 7 (Atoh7), Oligodendrocyte transcription factor 2 (Olig2), Ascl1 and Neurogenin 1 (Ngn1) [48, 67–69, 81, 86, 103, 143–147].

Stem cell characteristics have also been identified in chick and mouse Müller glia, including expression of Notch, Delta-like 1 (Dll1), Notch1, Nestin, Ceh-10 homeodomain containing homolog (Chx10), Forkhead box N4 (FoxN4), Pax6, Ascl1a and Hairy and enhancer of split 5 (Hes5) [71, 72, 76, 148–152] with human glia showing re-activation of Sox2 and Pax6 [153]. More similarities are continuing to be discovered, with a recent serial analysis of gene expression (SAGE) study identifying 61 out of 63 mature glia transcripts being common to progenitors [154] and profiling of 167 individual cells from mouse retina revealing even more glial transcripts [119, 155].

Qin and colleagues recently performed the first glia-specific microarray study (using fluorescence activated cell sorting (FACS) sorted GFAP:GFP glia), describing over 953 transcripts differentially regulated after retinal injury, the interaction of some of which are starting to be identified (Fig. 3.3b). Progenitor markers commonly expressed in the CMZ were identified including Ascl1, Sox3b, Sox4a [122]. The chaperones Heat shock 60-kDa protein 1 (Hspd1) and Monopolar spindle 1 (Mps1) are both reactivated during regeneration [122]. Hspd1 is expressed early on in dedifferentiating Müller glia where it drives proliferation and formation of neurogenic cell clusters, whereas Mps1 is specifically expressed later in proliferating photoreceptor progenitors, suggesting transient expression of different progenitor markers during different stages of regeneration [122].

Recent evidence for the role of epigenetics comes from identifying a role for the upregulated apobec2a and apobec2b cytidine deaminases, which act in a positive feedback loop with Ascl1a and are also necessary for proliferation [156].

The progenitor marker Pax6, which is expressed in low numbers of proliferating progenitors during persistent adult rod neurogenesis, is upregulated in a large number of progenitors during regeneration [48, 62]. In zebrafish, the Pax6b paralogue turns on 51 hpi and Pax6a 4–6 days post-light lesioning injury [157]. Mice in vitro culture studies have directly associated loss of Pax6 in adult with the reduction in retinal stem cell proliferation [158]. In zebrafish, Pax6a and b are necessary for initiating and maintaining mid and late Müller glia-derived progenitors as the knockdown of either causes reduced proliferation without affecting Müller glia cell cycle re-entry [157] (Fig. 3.3c). Müller glia-derived progenitor expansion peaks between 4 and 6 dpi [159] when these late stage progenitors express the proliferation markers Pax6a as well as Rx1 and Vsx2 [48].

While many factors are comparable, it is important to note that some genes and secreted factors involved in retinal cell generation may differ between embryogenesis and later stage growth/regeneration. Evidence for differences even between

embryonic and larval progenitors was identified in a genetic screen of 18 zebrafish mutants with normal embryonic eye development, but disrupted larval growth [3, 160]. Since even larval progenitors show some differences to embryonic progenitors, it will be interesting to determine, whether additional differences exist in the gene expression of adult regenerating Müller glia.

3.5.5 Division Mode

Beyond gene expression, studies are also investigating other stereotypical features to understand how similar to or different from progenitors regenerating Müller glia are. Will Müller glia always divide asymmetrically during regeneration or do symmetric cell divisions also contribute? Whether division recapitulates the mechanisms described during development [161] and how the composition of the resulting clones generated by each activated Müller glia compares to those obtained at different developmental stages remains poorly characterised. The mode of division during regeneration in retinal injury models and how it compares to development is heavily discussed, yet not completely understood.

Supporting evidence is mounting that asymmetric division mode might be a common and important mechanism by which a proliferative pool can be maintained at the same time at which retinal neurons are regenerated. After Müller glia activation and cell cycle re-entry, the first cell division has been described to give rise to BrdU/GFAP double-labelled siblings. One of these daughter cells retains Müller glia nuclear morphology and the other displays photoreceptor characteristics such as the expression of Cone rod homeobox (Crx) [60, 103, 122]. The non-glial progenitors remain associated and have been proposed to migrate along the radial processes of the sister Müller glia, as they become restricted to a specific neuronal cell fate to end up in their appropriate retinal layer and undergo terminal differentiation [60, 66, 85]. Interestingly, many proliferative Müller do not progress to this stage, even in fish, where only 30 % of BrdU-labelled cells are still present 2 weeks after injury [66]. The signals responsible for this difference within these cells or the environment remain poorly understood.

3.5.6 Progenitor Migration

During developmental retinogenesis, progenitors additionally undergo stereotypical migration modes, which can be compared to activated Müller glia during adult regeneration. During embryogenesis, proliferating retinal progenitor cells extend cytoplasmic processes from the external to the internal limiting membrane of the developing retina to form a pseudostratified neuroepithelium [162]. The nuclei of these progenitors move between the apical and basal sides as they undergo various stages of the cell cycle, in a process termed interkinetic nuclear migration (IKNM) [163–165]. Some data in teleosts are consistent with adult-regenerating progenitors

undergoing similar behaviour. Within a few days of laser ablation or excision injury in goldfish and zebrafish, Müller glia nuclei expressing glial markers are observed in all retinal layers, mimicking the distribution of retinal progenitor nuclei during development [65, 67]. Evidence for mitotic figures in the outer limiting membrane is consistent with IKNM [54]. However, unlike during development, proliferating dedifferentiated (GFAP negative) cells are closely associated with the processes of GFAP-positive glia cells, suggesting migration along processes of non-activated Müller glia as described above [85]. At the end of the proliferative phase, retinal progenitors during development undergo different modes of migration to their final laminar destination dependent on the neuron type [166]. Whether these are recapitulated by regenerating neurons remains unknown. Regenerated Zn5-labelled ganglion cells observed in the INL, IPL and GCL 7–11 days post-stab injury in zebrafish are consistent with laminar migration after fate determination [66]. The molecular factors that control these processes during regeneration have not been studied.

3.5.7 Extrinsic Factors Driving the Proliferative Phase

Extrinsic signals from the injury site are also very important for controlling the proliferative phase. These include many of the common signalling pathways that are implicated in developmental regulation (Fig. 3.3b). Dying cells must release extrinsic signals that allow Müller glia to sense sustained damage, such as TNFα released by apoptotic cells as described above.

Wnt signalling components are upregulated after injury and play an important role in balancing and maintaining proliferation. Both hyperstimulation (inhibiting retinal differentiation) or inhibition (premature differentiation with too little proliferation) negatively impact regeneration [114, 167]. Wnt signalling is one of the central pathways during the regenerative glial response. For example, Müller glia dedifferentiation and progenitor formation can be driven by inhibiting glycogen synthase kinase-3β (GSK-3β), a known inhibitor of Wnt signalling [167]. Also, injury-dependent induction of Ascl1a activates Wnt signalling by suppressing the Wnt inhibitor Dickkopf (Dkk1b) and inducing the Wnt ligand Wnt4a and receptors such as Frizzled 2 (Fzd2) [167]. The asymmetric division mode by which activated Müller glia generate one proliferative progenitor, while maintaining the other daughter as a Müller glia cell, also depends on Wnt signalling. Without Wnt, both daughters from the initial mitotic division accumulate in the ONL [114]. Similarly, in rats and mice, components of the Wnt pathway (Wnt3a, β-catenin) promote proliferation of neurospheres by leading to an increased expression of cell cycle genes including cyclin D1 [76, 168]. In chick, Wnt signalling regulates the Notch pathway (via Hairy1) to mediate progenitor maintenance at the CMZ [169].

Thus, Notch signalling interacting with Wnt is also involved in this response. Consistently, members of the Notch-Delta signalling pathway such as Notch1 and

Notch3 are upregulated in proliferating cells that co-express retinal progenitor markers after heat-induced injury in zebrafish [48].

The hedgehog pathway also functions to drive proliferation during development. For example, during chick development, inhibition of sonic hedgehog (shh) by cyclopamine results in inhibition of proliferation [170]. In 72 hpf Shh zebrafish mutants, the cell cycle exit is delayed, suggesting a role for hedgehog signalling as well [171]. In *Xenopus*, Hh also regulates the speed of cell cycle and cycle exit. Embryonic Hh overexpression leads to premature cell cycle exit and subsequent reduction in eye size, while Hh inhibition results in slower division and delayed cell cycle exit [172]. In postnatal mice heterozygous for Patched receptor mutations (Shh signalling), the retina contains a persistent zone of proliferating cells, which resembles the CMZ in lower vertebrates. This zone has increased proliferative activity in a retinal degenerative background [170]. Upregulated Hedgehog signalling also correlates with an increase in proliferation in the ciliary body of the retinitis pigmentosa mouse model from 30 days postnatal development [173], suggesting that this embryonic signalling pathway may also be involved in a regenerative response in a pathological background [37].

Purine nucleotides have been identified to regulate clonal expansion in vertebrates [174–176]. In zebrafish, endogenous adenosine diphosphate (ADP) nucleotides act as a crucial extrinsic signal through the P2Y1 purinergic receptors to regulate proliferation during retinal regeneration, possibly as a result of adenosine triphosphate (ATP) dephosphorylation from dying cells [177]. ADP analogues activating P2Y1 receptors in non-injured retinas also increase proliferation, while antagonists block proliferation and increased cell death.

Ciliary neurotrophic factor (CNTF) can act via multiple intracellular signalling pathways to influence different aspects of regeneration. While being involved in neuroprotection through a Mitogen activated protein kinase (MAPK)-dependant pathway, CNTF also acts in activated Müller glia through Stat3 to stimulate glia proliferation [178].

Müller glia themselves also contribute directly to relevant signalling pathways. One hour after damage, proliferating BrdU-labelled Müller glia positively regulate glial dedifferentiation by secreting heparin-binding epidermal-like growth factors (HB-EGF). HB-EGF acts through the EGFR/MAPK signal transduction cascade to activate genes associated with retinal regeneration [135]. Activation of HB-EGF by processing to shed its ectodomain is necessary and sufficient to stimulate Müller glia dedifferentiation into proliferating multipotent progenitors [135]. Suppression of HB-EGF by Insulinoma-associated 1a (Insm1a) restricts the zone of activated Müller glia [159]. Upon insm1a knockdown, HB-EGF expression is upregulated and acts upstream of Wnt/β-catenin signalling to promote generation of Müller glia-derived progenitors more distant [135, 170]. The knockdown of HB-EGF furthermore reduces progenitor expansion by decreasing proliferating Müller glia-derived progenitors with no change in cell death count [135].

Thus, it is clear that complex interactions between various signalling pathways are carefully orchestrated to generate the appropriate regenerative response (Fig. 3.3b) [115].

3.6 Müller Glia-Derived Regenerated Retinal Cells

3.6.1 Cell Cycle Exit and Differentiation

Regenerating progenitors, like their developmental counterparts, must eventually stop proliferating to differentiate and generate new postmitotic cells. Though the early activation and proliferation response of Müller glia is remarkably similar in different injury models, the timing and stages of neural differentiation depend highly on the injury model and cell types that are damaged in the first place. Recent work has started to identify the role of various factors during this phase (Fig. 3.3c, d).

Factors identified by microarray and subsequently shown to be important for this phase include midkine a and b (heparin-binding growth factors). Both midkines are upregulated during the proliferation and differentiation of photoreceptor regeneration in Müller glia and some neurogenic progenitors [179]. Midkine a is necessary for cell cycle progression and timing of cell cycle exit, although it is not sufficient to affect cell cycle exit when overexpressed [180, 181]. During development, midkine a acts via Inhibitor of differentiation 2 (Id2a), a transcription repressor that controls Notch signalling to regulate proliferation versus differentiation [182].

The Notch signalling pathway has been implicated in regulating fate choices to either allow clonal expansion or neural differentiation after retinal damage in fish. Activated Notch signalling markers such as Notch3 and Delta are elevated 7 days post-mechanical injury (small excision of the dorsal retina) and maintained until 21 dpi [183]. This timing overlaps with the late stages of progenitor expansion suggesting possible involvement in regulating progenitor proliferation and cell cycle progression [48, 67, 183]. The inhibition of Notch by the neuronal marker Atoh7 has recently been shown to promote differentiation in mammalian stem cells, suggesting that similar signalling mechanisms can act as major players in maintaining proliferation and preventing premature differentiation of progenitors across different vertebrate species [184].

Insm1a, described above to play a role in glia activation, also regulates the cell cycle and neuronal differentiation. After injury, Insm1a becomes restricted together to the injury site at 2 dpi [159]. Insm1a expression becomes localised to progenitors and peaks between 4 and 6 dpi. Increased expression of Insm1a in these progenitor cells suppresses cycling genes such as ccnb1, ccnd1, cdk1, cdk2, thus promoting cell cycle exit. Knockdown of Insm1a at 4 dpi suppresses the cell cycle inhibitor cdkn1c and results in an increased progenitor population.

3.6.2 Neural Differentiation

Following cell cycle exit, regenerating cells must differentiate to form new neurons and Müller glia (Fig. 3.3d). Differentiation towards specific cell types can be driven intrinsically by fate determinant factors, many of which are expressed during

development. In mouse explants for example combinatorial expression of Math3, NeuroD with or without Pax6 and Six3 can generate rods or amacrine cells [185]. The type of injury determines which neurons are affected. This may in turn influence this differentiation stage, for example by resulting in distinct extrinsic factors released at the injury site. In mouse, after NMDA treatment, regenerating neurons generate amacrine cells, expressing calretinin, NeuN, Pax6, Prox1 and GAD67-GFP, thus biasing regenerating cells specifically towards the missing cell type [152]. Use of kainite and colchicine, which destroy ganglion cells, versus NMDA, which does not, was shown to specifically result in regeneration of neurons with ganglion cell type gene expression and morphology [70]. In zebrafish, evidence for cell type-specific regeneration is accumulating. Light damage of photoreceptor, for example, causes regenerating cells to be biased to differentiate towards photoreceptors [85, 101]. The signals that instruct Müller glia to generate specific neurons can be very specific and can bias regeneration even towards a specific subtype of photoreceptor [186]. However, in other injury models glia are biased towards a specific cell fate, such as photoreceptors (influenced by Wnt, Shh, α-AA signalling), even though other cell types are damaged [3]. The possibility that endogenous cells within the injured retina or exogenous cells, such as cultured stem cells, may be able to recognise specifically which retinal neurons need to be replaced from the injured microenvironment is enticing. Characterisation of different injury models affecting different types of neurons need to be continued to investigate to what extent these extrinsic factors can influence cell fate decisions.

Microarray studies have allowed us to identify a range of upregulated transcription factors that control cell cycle exit, differentiation and fate specification. For example, Olig2 and Atoh7 are expressed at 68 and 96 hpi, respectively, in light lesioned zebrafish, probably reflecting the necessary upregulation of these genes for the differentiation into new photoreceptors. Results from these microarray studies are backed up by functional studies, showing for example that Atoh7 in rat drives ganglion cell regeneration [184]. A potential zebrafish orthologue of the photoreceptor-specific nuclear receptor gene NR23 is also expressed at 96 hpi, similar to that of Atoh7. In addition, microarrays can identify potential retinal regeneration genes in zebrafish that are already known to be important in other animal models. For example, thyroid hormone receptor β (Trb), which is crucial for normal rodent green cone photoreceptor development in microarray studies, is upregulated at 68 hpi in zebrafish [103]. Similarly, Crx is upregulated as Pax6 becomes downregulated during teleost regeneration (Fig. 3.3d) [60].

A large body of evidence suggests that the cellular and molecular events involved in differentiation during regeneration are remarkably similar to those observed during development [43, 44, 48, 179, 187]. However, the timing of neural differentiation during adult regeneration is much slower than during development. Whether this is a true difference between neural progenitors in development and Müller glia-derived progenitors, or whether the changed environment influences timing remains unknown. Studies of regeneration timing in different paradigms are being used to assess whether molecular expression and fate determination mirrors the sequence observed during experiment. For example, we can compare the regeneration of early and late born retinal neurons. Ouabain, which ablates all neurons at high

concentrations, can be used at low local concentrations to selectively affect ganglion cells. Selective loss of ganglion cells (the firstborn neurons during development) results in differentiation of newly generated ganglion cells already at 7 dpi [86]. These regenerating ganglion cells first transiently express Atoh7, Pax6 and Zn5, which are associated with developmental ganglion cell fate specification and axonal outgrowth, followed by late differentiation markers such as HuC/D (at 14 hpi) and full retinal lamination is fully re-established by 60 hpi [86, 87]. Interestingly, higher concentrations of ouabain, which destroy other retinal neurons as well, re-establish normal layering also by 60 hpi. This indicates that the timing of the overall regeneration is unchanged by the increased damage or/and involvement of additional retinal neuron types [86].

By contrast, photoreceptors (later born during development) lost in light damaged retinas return to their normal density already by 28 dpi [85]. Whether this is in part due to the involvement of photoreceptor precursors additional to glia cells or differential response by Müller glia remains open. As photoreceptors are usually generated after ganglion cells in development, it is interesting to speculate that regeneration of specific cell types may not need to progress through the same gene expression and lineage sequence that is so reproducible during development.

The relative spatiotemporal expression patterns of Atoh7, Pax6, Islet-1, Hu and Neurolin in regenerating retina parallel their expression pattern during retinogenesis in the developing zebrafish. However, some developmental markers such as Brain-specific homeobox 3b (Brn3b), Fibroblast growth factor 8 (Fgf8) and shh [188–190] are not re-expressed after ouabain-induced ganglion cell loss [87, 136]. This difference may be due to the different microenvironment generated in these injury models.

Additionally, regeneration may involve processes that are not required during development. For example, β-galactoside-binding protein galectin 1-like 2 (Drgal1-L2) is usually only expressed in the notochord of the developing zebrafish, not in the retina (Vasta 2004). However, retinal progenitors of light lesioned zebrafish retina secrete Drgal1-L2, a knockdown of which causes a reduction in the number of regenerated rods without affecting proliferation [191]. Thus, although the microenvironment generated after retinal injury is thought to resemble embryonic stem cell niches [48], molecular differences do exist. Additionally regenerating photoreceptors while becoming functional fail to reconstitute the mosaic pattern that is established in larva during the late developmental phase [85], suggesting that regeneration cannot completely recapitulate retinogenesis during development. How these differences affect the regenerative response in terms of number of cells differentiated, re-integrated and functioning needs to be determined.

3.6.3 Müller Glia Differentiation

Müller glia themselves must also be maintained or regenerated, due to their important role during adult retinal function (Fig. 3.3e). Because Müller glia markers such as GFAP are initially downregulated as glia re-enter the cell cycle, but recover by 17

days post-light lesioning, Müller glia are believed to regenerate [69]. The dedifferentiation and redifferentiation of Müller glia-derived progenitors into mature Müller glia can be observed by following expression of Ngn1. Ngn1 is absent from the adult zebrafish retina, but becomes upregulated in Müller glia around 72 hpi. As these Müller glia dedifferentiate, they loose their glial markers, but start being labelled with Ngn1. Subsequently these same Ngn1-labelled cells start re-expressing glia markers as some of these regenerating cells regenerate mature Müller glia [103].

Some of the pathways important in early stages as described above also play roles at these later differentiation stages. For example, disregulation and enhanced TGFβ signalling has drastic consequences, inhibiting proliferation and driving glial fate at the expense of neurogenesis [137]. Insm1a not only drives cell cycle exit in differentiating neurons, but HuC/D immunostaining also showed that differentiated neurons were rare in Insm1a knockdown retinas at 6 dpi, revealing a role of Insm1a as a driver of neuron differentiation [159].

3.6.4 Survival and Functional Integration

Characterising the recovery of lost cell number and general retinal morphology has been the main method of assessing retinal regeneration. With the advent of non-invasive techniques, this can also be carried out to some extent in living adult animals including zebrafish. For example spectral-domain optical coherence tomography can be used to quantify retinal layer thickness, making use of differential light scattering properties of the nuclear and synaptic retinal layers [192]. The final outcome and ultimate measure is the restoration of functional vision. Newly generated neurons must also survive, regenerate axons to appropriate targets and functionally integrate into existing neural networks. The delay observed between histological retinal restoration and behavioural recovery is believed to be due to the long-range axonal navigation [87].

A defining factor of regeneration of ganglion neurons is the regrowth of their long axons. These axons connect the retina to their primary visual targets in higher brain areas and are also crucial for ganglion cell survival. Teleosts such as goldfish exhibit a broad capability to regenerate their axons [193], which survive in the absence of glial scar formation [194]. By contrast mammalian axon regeneration is significantly reduced, being inhibited by myelin and scar formation [195–198]. Regenerated axons in teleosts undergo successful pathfinding to their target sites, though some of them do follow wrong projection pathways during regeneration as compared to development [199, 200]. In teleosts, axons successfully project to at least one of the ten different target sites in the diencephalon and mesencephalon, which are re-innervated by 4–6 weeks after optic nerve lesioning [194]. After re-innervation, refinement occurs via signalling pathways such as ephrins and Eph receptors [201].

In parallel with refinement and reorganization of axonal connections vision is gradually restored. This can be measured with various methods including electrophysiology and behavioural output. The electroretinogram (ERG), which evaluates

retinal function, has been used in many models, including embryonic and adult zebrafish [202] to follow degeneration and regeneration. As in mammals, a recording electrode is placed onto the cornea of the zebrafish and the electrical output generated from retinal neurons is assessed by three distinct waveforms by inducing flashing light, while changing factors such as light intensity and duration [203]. Functional recovery of photoreceptor activity in goldfish [204] and rainbow trout [205] has been measured using ERGs. Additionally methods available to monitor functional recovery include visual-driven behaviour tests in zebrafish [87] including optokinetic nystagmus [206], dorsal light reflex [204, 207] and escape response [208]. For optokinetic response or nystagmus, teleost eye movements are tracked in response to moving grating stimuli [206, 209, 210]. This method was used to show that ouabain-induced retinal damage and subsequent ganglion cell regeneration resulted in functional recovery comparable to control animals by 98 hpi. Future investigations into the potential of different strategies must integrate these functional long-term assessments, as functional recovery represents the ultimate goal of regeneration.

3.7 The Role of Extrinsic Cues During Retinal Development and Regeneration

In addition to gene regulation being functionally conserved between development and adult growth/regeneration, there is mounting evidence that this conservation is also observed with extrinsic factors, including the presence of growth factors and the microenvironment at the injury site. For example, embryonic Müller glia transplanted into adult chick retinas fail to differentiate into neurons, suggesting that the aging environment no longer supports this process [70]. Excitingly, BrdU incorporation in mouse can be increased by growth factors, showing that identification of external factors present a feasible avenue for increasing regeneration [152, 211]. Consistently, data from murine studies also show improved integration of regenerated neurons is improved in diseased retina, revealing the importance of environmental signals [212].

In response to injury, many types of growth factors are produced to improve the response during the different phases we describe here, including Fgf [213–217]. In zebrafish, Fgf signalling is crucial for eye patterning and normal morphogenesis during development and regeneration of neurons after lesioning. Fgf signalling is necessary for the maintenance of differentiated photoreceptors as inhibition of Fgf signalling results in rapid photoreceptor degeneration and disorganisation [218, 219]. During retinal regeneration, inhibition of Fgf signalling results in a reduction in proliferation after light lesioning [218] and intravitreal Fgf injections contribute to increased rod progenitor proliferation [219]. The Fgf pathway is similarly involved in amphibian and chick retinal regeneration [220, 221]. Other signalling pathways and secreted peptides involved in vertebrate retinal regeneration, some of which have been discussed in this chapter, include Shh [148, 221, 222], Wnt [114],

Insulin growth factor (IgF) [223, 224], EGFR [135], Notch [225], bone morphogenetic protein (BMP) [226, 227] and Jak-Stat [103], taurine [228], retinoic acid [229], neurotrophin factor 3 [230] and CNTF [178, 214, 216, 231].

Some of these signalling pathways are now being tested in other animal models. The introduction of extrinsic factors in optic nerve crush injured mice improves survival and axonal regeneration and prevents further neuronal damage [232]. Intravitreal injection of constitutively active Ras-related C3 botulinum toxin substrate 1 (Rac1) Rho-related small GTPase protects ganglion cells from injury-induced death in vivo, permitted the elongation of axonal outgrowths to their target site and prevented axonal degradation. In addition, intraocular injections of CNTF and BMP4 into chick prior to toxin-induced injury reduces the death of amacrine and bipolar cells [233]. α1-tubulin is upregulated in retinal ganglions cells and was shown to be necessary to regenerate axons after damaged optic crush injury in zebrafish [81]. In vivo, *tuba1a* mRNA knockdown after optic crush results in a suppressed regeneration of ganglion axons without increasing cell death [234]. These studies show the potential of using rapidly gained information from the zebrafish system to test the capacity to influence these processes in higher vertebrates.

3.8 Concluding Remarks

For successful progress, therapeutic approaches must continue to target all aspects of retinal diseases/disorders including prevention or slowing of disease, enhancing neuroprotection and survival of existing neurons, as well as improving regeneration of endogenous cell sources and regeneration driven by exogenous stem cell sources.

Past studies have drawn on the particular strength of the vertebrate zebrafish system for expanding our knowledge on the signalling pathways involved during retinal development, identifying factors differentially regulated during the highly regenerative response after retinal injury and performing functional loss and gain of function studies for candidate genes. The zebrafish retinal regeneration field has contributed towards our understanding of how the resident glia cells are activated, how they dedifferentiate to give rise to progenitor-like cells that are similar, yet not identical to developmental progenitors, how proliferation and cellular expansion are controlled and how newly regenerated neurons and glia finally exit the cell cycle and differentiate. Here, we summarised some of the recent signalling pathways and their role at different regenerative stages.

Given the potential of mammalian Müller glia to respond to injury, activate some regenerative response, re-enter the cell cycle and differentiate into various neurons either in vivo or in vitro, the goal now remains to improve each of these steps and survival and integration of regenerated neurons. As the retina is one of the main organs currently in clinical trials for stem cell-based therapy [38], processes uncovered during Müller glia-driven zebrafish regeneration will be beneficial to improve these approaches. Identified signalling pathways can be used to drive differentiation of stem cells exogenously in culture. No drastic differences

have been identified between the highly regenerative versus the vertebrate species, with more limited regenerative capacity. This is in line with the general conservation in anatomy, function and molecular developmental mechanisms across all vertebrates. Thus, with the genetic similarities discovered during development of vertebrates from zebrafish to humans, improving the existing Müller glia response in mammals by addition of combinations of pro-regenerative factors that are so rapidly being discovered in the zebrafish represents a realistic goal. As this work continues into the future in parallel with mammalian studies that assess similarities, differences and limitations between the systems, the hope is that Müller glia-derived regeneration can be improved in higher vertebrates.

Acknowledgments We are extremely grateful to Alexandra D. Almeida, Ryan MacDonald, Florence D'Orazi and Ashley L. Siegel for comments on this chapter.

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Chapter 4
Stem Cells and Regeneration in the *Xenopus* Retina

Magdalena Hidalgo*, Morgane Locker*, Albert Chesneau, and Muriel Perron

Contents

Abbreviations

BMP	Bone morphogenetic protein
BrdU	5-Bromo-2′-deoxyuridine
CMZ	Ciliary marginal zone
DNA	Deoxyribonucleic acid

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A. Pébay (ed.), *Regenerative Biology of the Eye*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4939-0787-8_4,

EdU	5-Ethynyl-2′-deoxyuridine
EFTF	Eye field transcription factors
ERK	Extracellular signal-regulated kinases
FGF	Fibroblast growth factor
GFP	Green fluorescent protein
Hes4	Hairy and enhancer of split 4
iCasp9	Inducible caspase 9
MEK	Mitogen-activated or extracellular signal-regulated protein kinase
Mtz	Metronidazole
NTR	Nitroreductase
Pax6	Paired box protein 6
Rax	Retina and anterior neural fold homeobox gene
RPE	Retinal pigmented epithelium
RPE65	Retinal pigment epithelium-specific 65 kDa protein
RVM	Retinal vascular membrane
shRNA	Small hairpin RNA

4.1 Introduction

4.1.1 The Remarkable Regenerative Capacity of the Urodele Amphibian Retina

It is in the mid-eighteenth century that the Swiss naturalist Charles Bonnet observed the process of regeneration in many species including worms, hydras, starfish, snails, crayfish, and amphibians. He discovered that the newt (an urodele amphibian) could regenerate its eyes when small parts were removed [1]. A 100 years later, Philipeaux [2], Griffini and Marcchio [3], Colucci [4], and Wolff [5] highlighted that the regenerative capacity of the newt retina was actually much broader since it could reform even after entire ablation. This led to further investigations in the twentieth century, aimed at documenting the cellular sources contributing to this process. An unambiguous candidate, that can efficiently transdifferentiate upon retinectomy, proved to be the retinal pigmented epithelium (RPE), the cell layer overlying the neural retina [6–13]. Besides, new retinal neurons can also originate from the ciliary margin, a small peripheral region of the adult eye that contains mitotically active retinal cells [6, 9, 12, 13]. Although urodeles were prominently used as models for experimental embryology [14], they did not really move into the genomics and reverse genetics era of the twentieth century. Consequently, the molecular mechanisms underlying their acknowledge regenerative retinal capacities remain largely unexplored.

4.1.2 Xenopus laevis *as a Novel Model System for Retinal Regeneration Studies*

In the 1950s, the South African clawed frog *Xenopus laevis*, an anuran amphibian, was favoured by molecular and developmental biologists and ultimately supplanted the newt. Although its ability to regenerate organs is much less pronounced than that of urodeles, it also recently emerged as a leading model for regeneration research [15]. Regarding the retina, it was long known that regeneration could occur in larvae after resection of up to two-thirds of the eye [16–22]. This potential was however thought to disappear after metamorphosis [23]. Yet, adult *Xenopus* RPE proved as plastic as the newt one. It could indeed reform a new retina when grafted in a host posterior eye chamber [24–26]. In 2007, Yoshii et al. therefore re-investigated this issue and demonstrated that *Xenopus* can actually regenerate its retina at post-metamorphic stages following retinectomy [27]. In this chapter, we review recent progress in the field focussing on the resurgence of *Xenopus* as a model system for retinal regeneration studies. Given the potential applications for regenerative medicine, we will highlight the opportunities offered by this model to uncover the underlying cellular and molecular mechanisms.

4.2 Retinal Regeneration in Amphibian via RPE Transdifferentiation

4.2.1 *RPE Is a Major Cellular Source for* Xenopus *Retinal Regeneration*

The RPE is a monolayer of pigmented epithelial cells located in between the neural retina and the choroid where it forms the outer blood–retinal barrier. It is endowed with multiple essential functions for the visual process including transport of nutrients, ions and water, absorption of excess incoming light, phagocytosis of shed photoreceptor membranes and protection of the retina structural and physiological integrity. As in urodeles, Yoshii and collaborators found that the RPE constitutes a major cellular source for de novo production of retinal cells in *Xenopus* [27–29]. This occurs through a transdifferentiation process where RPE cells dedifferentiate, undergo several rounds of cell divisions and eventually give rise to all types of retinal neurons. Two series of arguments favour the hypothesis that at least part of the regenerating retina has an RPE cell of origin. First, it stains positive for RPE65, a specific marker of RPE cells [26, 27]. Second, grafting green fluorescent protein (GFP)-labelled RPE into a retinectomised eye results in a newly formed neural retina that also expresses GFP [30].

Of note, the amphibian RPE neurogenic potential probably finds its source in a common embryonic origin shared with the neural retina. Both indeed derive from ocular precursors with bi-potential competence of the young optic vesicle and it is only at the optic cup stage that both fates become determined [31]. Clearly however, even in the adult, the amphibian RPE remains plastic, which allows it to transdifferentiate into neural retina following damage. In contrast, except in pathological situations, this property is limited to a restricted developmental window in birds and mammals [31, 32]. Whether this is due to an intrinsic repression of the retinal program in RPE cells and/or to extrinsic constraints from the retinal environment is presently unclear.

4.2.2 *Role of the Retinal Vascular Membrane for RPE Cell Transdifferentiation*

Interestingly, the transdifferentiation process occurring in *Xenopus* differs from that previously described in the newt. In the latter, RPE cells lose their cellular junctions, start proliferating, undergo depigmentation and locally form a bilayered structure consisting of both presumptive RPE and neural retina [32]. In contrast, *Xenopus* RPE cells do not transdifferentiate inside their original site [27]. Instead, a subpopulation detaches from the RPE monolayer and migrates through the vitreous onto the retinal vascular membrane (RVM) where it forms a novel neuroepithelium (Fig. 4.1). Similar behaviour has been described as well in the anuran amphibian, *Rana catesbienna* [33]. The RVM, which constitutes the inner limiting membrane of the retina, consists of a basement membrane and numerous blood capillaries. It is assumed that its persistence following retinectomy is a *sine qua non* condition for regeneration to occur [27]. This could be the reason why previous studies were unsuccessful to reveal the regenerative potential of the post-metamorphic *Xenopus* retina. In line with this hypothesis, Yoshii and collaborators observed that when the RVM is intentionally removed, the retina does not regenerate [26, 27, 32]. The molecular nature of the RVM that provides such permissive conditions for RPE cell transdifferentiation remains to be investigated. A potential candidate originally identified in *Rana catesbienna* is laminin, a major extracellular matrix component, since its blockade through intraocular injection of a specific antibody inhibits retinal regeneration [34–36].

4.2.3 *Influence of the Choroid on RPE Cell Transdifferentiation*

Besides this differential requirement for RVM between newt and *Xenopus*, a common feature that emerged from several studies is the essential role played by the choroid in the regeneration process. This tissue is the vascular layer of the eye and is separated from the RPE by an extracellular matrix called the Bruch's membrane. RPE/choroid interaction was mainly analysed in organotypic cultures [27, 37–39]. In this system,

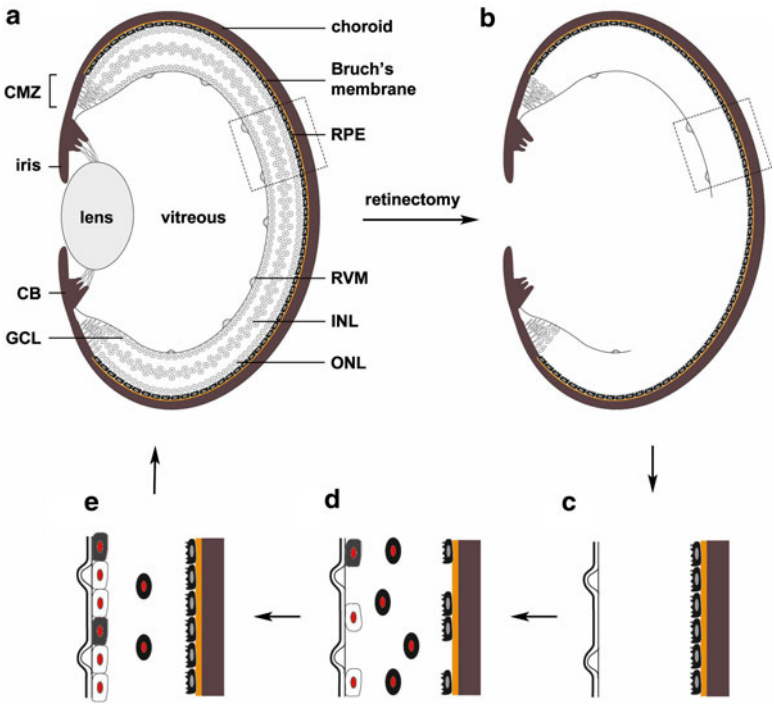

Fig. 4.1 Retinal regeneration via transdifferentiation of the RPE in *Xenopus laevis*. Following retinectomy (**b**, **c**), some RPE cells detach from Bruch's membrane, start expressing Pax6 (*red*), migrate and attach to the remaining RVM, where they form a new epithelium (**d**). RPE cells anchored onto the RVM initiate proliferation and transdifferentiate into neural precursors (**e**). They finally regenerate the whole neural retina, while RPE that remained attached to the Bruch's membrane renews itself (**a**). *CB* ciliary body, *CMZ* ciliary marginal zone, *RVM* retinal vascular membrane, *RPE* retinal pigmented epithelium, *ONL* outer nuclear layer, *INL* inner nuclear layer, *GCL* ganglion cell layer. Adapted from [26, 32]

when both tissues are left assembled, numerous RPE cells rapidly migrate out of the explant and progressively acquire neuronal characteristics. This fails to happen when the choroid is removed but can be rescued by re-associating the two tissues with a membrane filter in between, suggesting the need for diffusible substances emanating from the choroid [27, 38] (see below Sect. 4.2.5).

More recently, new advances in RPE/choroid interactions were gleaned from a new culture method allowing for 3D reconstruction of retinal structures from *Xenopus* RPE. The explants are overlaid by a gel matrix as a substitute for the RVM to better mimic the in vivo situation [37, 40]. Under such conditions, when isolated sheets of RPE are cultured in the presence of appropriate growth factors (see paragraph below), a whole layered neural retina forms. In contrast, such regeneration does not occur when the RPE remains physically attached to the choroid [37, 40]. As a whole, these experiments thus suggest that transdifferentiation requires (1) alterations in cell-to-cell and/or cell-to-Bruch's membrane interactions and (2) diffusion of signals emanating from the choroid.

4.2.4 Reactivation of Eye Field Transcription Factors During Transdifferentiation

Pax6 is part of the "eye field transcription factors" (EFTF), which at the neurula stage determine the presumptive eye region and are characterised by their ability to induce ectopic eye formation following misexpression [41]. It is later expressed in the whole optic vesicle and then vanishes in the differentiating RPE at the optic cup stage. In newts as in frogs, reactivation of *Pax6* expression represents an early molecular change associated with RPE transdifferentiation [27, 42, 43]. Using organotypic cultures, Nabeshima et al. [40] further revealed that *Pax6* up-regulation, as well as that of *Rax*, another EFTF, crucially depends on the loss of adhesion with the Bruch's membrane. How loss of adhesion triggers these transcriptional changes remains unknown. Of note, *Rax* has been shown to be required for proper regeneration following retinal resection, using a transgenic shRNA-based approach in pre-metamorphic *Xenopus* larvae [44]. Together, such re-expression and involvement of EFTF suggest that RPE transdifferentiation involves a reprogramming event towards an embryonic-like retinal state. Further investigations, including transcriptomic analyses, are however required to determine to which extent transdifferentiating RPE cells are comparable to young stem/precursor cells of the optic field.

4.2.5 Growth Factor Implication in RPE Cell Transdifferentiation

The fibroblast growth factor (FGF)-mediated MEK-ERK signalling is an accepted "pro-retinogenic" core pathway known to be sufficient for fate switching of immature RPE into neural retina during embryonic development [32]. In line with this, among several tested growth factors, FGF2 was found the only potent inducer of *Xenopus* RPE transdifferentiation in vitro [34]. Mitsuda et al. further demonstrated in newt organotypic cultures that FGF2 addition could compensate for the absence of choroidal diffusible signals [38]. More recently, FGF2 ability to promote amphibian regeneration was assayed in vivo in *Xenopus* tadpoles, following complete removal of the neural retina, including the RVM [45]. As mentioned above, RPE transdifferentiation does not occur spontaneously under such conditions. However, an FGF2-soaked bead placed inside the retinectomised eye is sufficient to trigger the regeneration of a complete layered retina. This raises the question as to whether endogenous FGF pathway is actually required during RPE transdifferentiation. Vergara and Del Rio-Tsonis found that expression of FGF receptors 1 and 2 are strongly upregulated following retinectomy. This suggests that retina removal enhances responsiveness to FGF signalling. In addition, MEK-ERK signalling blockade was found to inhibit the regeneration process in both *Xenopus* and newt [45, 46], as previously reported in chick [47, 48].

Another question is whether and how FGF pathway is linked to the aforementioned reactivation of EFTF during RPE transdifferentiation. Kuriyama et al. [37] found that early up-regulation of Pax6 observed when RPE cells detach from the choroid still occurs when MEK-ERK signalling is inhibited. However, FGF2 seems to be needed for sustained expression of the transcription factor during the transdifferentiation process. This led to the proposal of a two-step model: an FGF-independent step where loss of interaction with the basement membrane leads to a reversible Pax6 induction and a FGF-dependent step further driving Pax6-positive RPE cells into neuronal progenitors [32, 37].

4.3 Retina Regeneration by Stem Cells of the Ciliary Marginal Zone

4.3.1 The CMZ Sustains Continuous Retinal Adult Neurogenesis

The ciliary marginal zone (CMZ), that lies in the peripheral region of the eye between the iris and the retina, is a specific structure of the fish and amphibian eye, only transiently found in post-hatched chicks and absent in mammals. It contains actively proliferating cells which contribute to continuous retinal growth throughout the animal life (Fig. 4.2a, b) [49–52]. Indeed, the use of birth date indicators such as ^{3}H-thymidine highlighted that new rings of retinal cells are constantly added from this zone. In addition, single cell lineage analysis revealed a broad range of clone sizes, strongly suggesting that the CMZ harbours at least two types of cells: self-renewing stem cells and progenitors that only undergo a limited number of

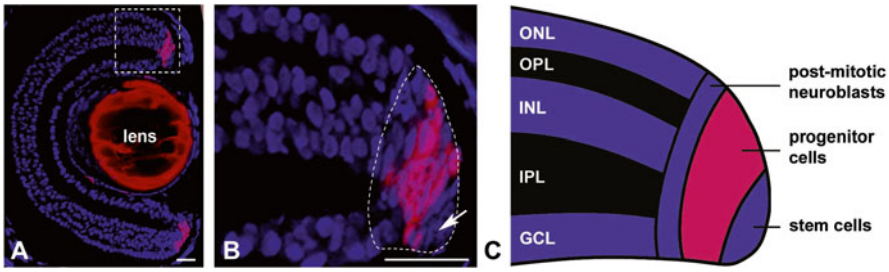

Fig. 4.2 Organisation of the *Xenopus laevis* CMZ. (**a**, **b**) Proliferative cells were labelled with EdU (*red*, 5 hr pulse). Nuclei are counterstained with Hoechst (*blue*). Non-specific staining is observed in the lens. (**b**) Magnification of the delineated region in (**a**) showing the CMZ (*dashed line*). Stem cells in the most peripheral region are EdU-negative due to their slow cell cycle kinetics (*arrow*). (**c**) Schematic representation of (**b**). *GCL* ganglion cell layer, *INL* inner nuclear layer, *IPL* inner plexiform layer, *ONL* outer nuclear layer, *OPL* outer plexiform layer. Scale bar = 25 μm

divisions [53]. The cell composition of the clones further indicated that CMZ cells are able to give rise to all types of retinal neurons and Müller glia [53]. Recently, elegant experiments based on long-term in vivo lineage analyses of individual labelled cells in fish firmly demonstrated that the CMZ indeed contains *bona fide* stem cells that self-renew and are multipotent [54].

Comparative analyses of gene expression patterns revealed that the spatial organisation of the *Xenopus* CMZ mirrors the temporal sequence of retinal development, with stem cells residing in the most peripheral margin, followed more centrally by progenitors and their post-mitotic progeny (Fig. 4.2c) [55–59]. Localisation of retinal stem cells in a geographically identified niche confers clear advantages to the CMZ as a model to study neural stem cells. However, not so far ago the number of available specific markers of these cells was still very limited [57, 60]. Recently, Xue and Harris [61] showed that the most peripheral cells of the CMZ can be distinguished as being positively labelled for *c-myc* and negatively for *n-myc*. Furthermore, short- and long-term EdU labelling revealed that these *c-myc*$^{+}$*/n-myc*$^{-}$ cells exhibit a specific proliferative behaviour compared to more central progenitors. They are notably characterised by self-renewal and low rates of division and thus likely correspond to the stem cell pool. To gain further insights into the molecular signature of CMZ retinal stem cells, we performed a large-scale in situ hybridisation screen in *Xenopus* and identified 18 novel markers specifically expressed at the tip of the CMZ [62]. Genes identified in this screen can be easily retrieved in the searchable database XenMARK [62–64]. Interestingly, analysing the developmental expression pattern of some of them, such as *Hes4* (that encodes a transcriptional repressor of the bHLH-O family), revealed new insights into the cell of origin of adult retinal stem cells, a yet unresolved issue in the field. Our results indeed suggest that they likely originate from a discrete population of cells located at the border between the presumptive RPE and neural retina at the optic vesicle stage [65].

4.3.2 The CMZ Contributes to Xenopus *Retinal Regeneration*

Although *Xenopus* regeneration is believed to be mostly RPE dependent, several lines of evidence support a contribution of the CMZ to retinal repair. Following retinal degeneration induced by devascularisation in *Rana castesbienna* tadpoles, both processes indeed take place simultaneously and a new retina is generated centrally from RPE cells and peripherally from increased proliferation of the CMZ [33]. Regarding post-metamorphic *Xenopus laevis*, Mitashov and Maliovanova [66] noticed that when the retina was removed from the eye, RPE transdifferentiation did not occur (probably, as mentioned above, because RVM had been removed as well). They nevertheless still observed partial regeneration likely originating from the remaining ciliary margin. In line with this, in the *Xenopus* model of retinectomy that leaves RVM intact, a non-RPE origin of some regenerating cells was

also suspected by the presence of non-pigmented RPE65-negative neuroepithelial cells at the periphery of the RVM, in close vicinity of the CMZ [26, 27]. In a less drastic lesional paradigm of punch biopsy that removes a transverse section of the eye, including the choroid, RPE and retina, increased proliferation and neurogenesis are observed locally at the site of injury but also more distantly within the CMZ [67]. Finally, the extent of CMZ contribution might be species-dependent. Contrasting with the situation in *Xenopus laevis*, the CMZ was indeed recently found to be the major cellular source for the regeneration process in *Xenopus tropicalis* after whole retinal removal [68].

The molecular cues that trigger *Xenopus* CMZ cell activation upon retinal injury remain largely unknown. We previously showed that Wnt and Hedgehog signalling are involved in the regulation of their activity in physiological conditions [69–72]. We further recently discovered that these pathways exert opposed and counterbalancing functions in the tadpole CMZ and negatively regulate each other activity [73]. Such an antagonistic interplay, with Wnt likely maintaining the proliferative pool and Hedgehog pushing it towards cell cycle exit, is believed to sustain homeostatic growth. An obvious question is whether injury might trigger an imbalance in their tight equilibrium that may enhance CMZ activity. Besides, candidate pathways, such as FGF and bone morphogenetic protein (BMP) signalling are worth to be tested since they were shown in the chick model to cooperate, together with Hedgehog signalling, to promote survival and proliferation of CMZ cells [74]. In addition, whether regeneration primarily involves changes in CMZ stem cell behaviour and/or impacts on the proliferative potential of progenitors also remains to be further examined.

4.3.3 Restoration of a CMZ in the Regenerating Neural Retina

Interestingly, it is likely that the CMZ can itself be regenerated, permanently or as a transient structure sustaining reformation of a new retina. After retinal resection of the nasal-dorsal quarter of the eye, Martinez-De Luna et al. nicely described that neuroepithelial cells repopulating the wound spatially organise similarly as CMZ cells [44]. Based on gene expression patterns, different zones can indeed be distinguished with a central to peripheral differentiation gradient. It is likely however that this CMZ-like structure lacks true retinal stem cells since it does not persist once the gap caused by resection has been refilled with new differentiated cells. In contrast, the CMZ might be able to regenerate in its endogenous location following ablation. In a model of retinectomy where both the retina and the CMZ are surgically removed and regeneration induced by FGF2, a novel CMZ forms in the margin of the RPE-derived new retina, as inferred by BrdU labelling and expression of the CMZ marker *Ddx39* [75]. Whether this regenerated CMZ possesses self-renewing retinal stem cells able to sustain continuous retinal growth remains however to be investigated.

4.4 Retina Regeneration via Müller Cells

Müller cells constitute the resident radial glia of the vertebrate retina. In fish, they can efficiently regenerate all types of neurons following retinal damage. This potential is much more limited in birds and even more in mammals. In the latter indeed, only a few Müller cells spontaneously re-enter the cell cycle upon injury [58, 76–80]. Where do amphibians stand with regard to Müller cell-driven retinal regeneration? Amphibian regeneration was mostly studied following retinectomy, thus preventing any investigation of a potential Müller cell involvement. In a newt model of retinal detachment however, some proliferating cells can be detected in the inner nuclear layer in addition to the RPE and CMZ. These are possibly Müller cells and they partially contribute to replace the damaged retina [81, 82]. In contrast, in a more recent model of conditional rod cell ablation (see below), although Müller cell hypertrophy was observed, preliminary experiments mentioned by the authors suggest no increased proliferation [83]. It is thus likely very different from the fish situation. However, it cannot be excluded that the occurrence of Müller cells recruitment during amphibian regeneration strongly depends on the type of lesion. We found indeed that *Xenopus* Müller cells can re-enter the cell cycle after a needle-stick retinal injury (our unpublished data; Fig. 4.3). This clearly needs to be further investigated to decipher in particular whether or not, in such conditions, they actually generate new neurons. If this were the case, this new lesional paradigm would be extremely valuable to study the molecular mechanisms underlying Müller cell reactivation.

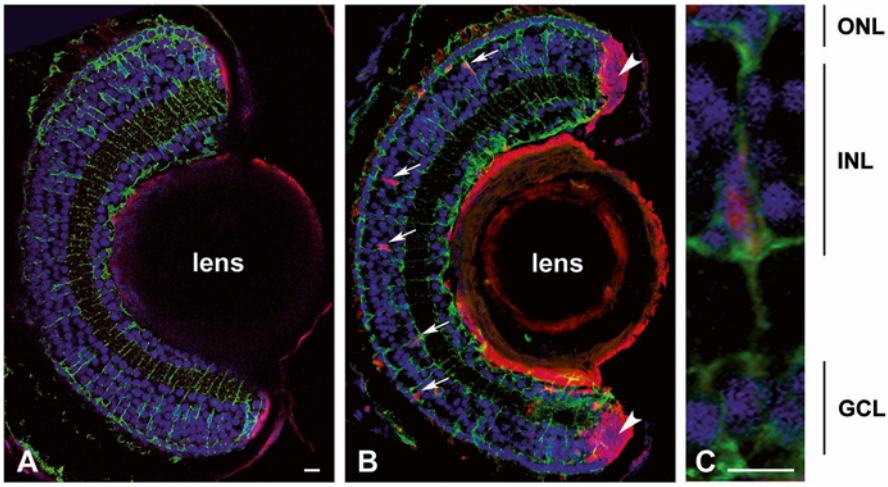

Fig. 4.3 CMZ cells and Müller glia proliferate following retinal injury. Sections were immunostained with anti-PCNA (*red*; proliferating cells) and anti-CRALBP (*green*; Müller cells) antibodies. Nuclei were counterstained with Hoechst (*blue*). (**a**) Control eye. (**b**) Needle-stick damaged eye. *Arrows* and *arrowheads* point to PCNA-labelled Müller cells in the central retina and CMZ cells in the peripheral region, respectively. (**c**) Magnification of a PCNA-positive Müller cell. *ONL* outer nuclear layer, *INL* inner nuclear layer, *GCL* ganglion cell layer. Scale bar = 10 μm

4.5 Transgenic *Xenopus* Models of Photoreceptor Cell Degeneration

Although fundamental and valuable information is undoubtedly gleaned from regeneration studies using retinal resection or retinectomy, such experimental paradigms are far from mimicking molecular and cellular events that occur in human retinal dystrophies. The development of transgenesis procedures in *Xenopus* [84] allowed the emergence of novel models to investigate the pathogenic mechanisms underlying retinal degenerative diseases. This was done in particular to study the molecular basis of Retinitis pigmentosa [85–89], a heterogeneous inherited disorder characterised by the initial loss of rod photoreceptors. Thirty percent of autosomal-dominant cases are caused by mutations in the rod opsin gene that encodes the protein moiety of the photoreceptor light-sensitive pigment rhodopsin. Transgenic *Xenopus* expressing the most prevalent rod opsin mutation P23H brought novel insights into a controversial story by highlighting that photoreceptor death likely occurs as a result of protein retention in the endoplasmic reticulum rather than by altered rhodopsin signal transduction [86, 87].

Another transgenic model of rod degeneration was also generated where the function of kinesin II, a motor protein involved in ciliogenesis, was compromised [90]. Such a model may offer opportunities to study a class of inherited conditions known as retinal ciliopathies, which result from dysfunction of the photoreceptor outer segment, a highly modified and specialised primary cilium [91].

Besides, transgenic *Xenopus* lines were recently developed to follow the regeneration process that accompanies cell type-specific ablation. To conditionally and selectively induce apoptosis of a targeted neuronal population, the Nitroreductase (NTR)/Metronidazole (Mtz) system previously developed in zebrafish was adapted in *Xenopus* [83, 92]. A transgenic line was generated that expresses *Escherichia coli* NTR under the control of the rod opsin promoter. NTR converts the prodrug Mtz into a cytotoxic DNA cross-linker that does not diffuse to neighbouring cells. Thus bathing tadpoles in Mtz at a given time leads to specific rod cell death [83]. Noticeably, the authors observed that this is progressively followed by cone degeneration [83]. This is of particular interest since this closely mimicks the situation observed in patients with retinitis pigmentosa. Because of its high cone/rod ratio, *Xenopus* is thus particularly suitable to investigate rod–cone interactions during retinal degeneration. Besides, since the Mtz can be washed away, the system is reversible, allowing for examination of subsequent photoreceptor regeneration. Indeed, EdU incorporation assay revealed that newborn rods are formed when *Xenopus* larvae are allowed to recover from the Mtz treatment [83].

Of note, seemingly contradictory results were reported using another paradigm of conditional targeted cell ablation [67]. In their study, Lee et al. used a modified caspase-9 (iCasp9) transgene placed downstream the *Xenopus* rod opsin promoter [93]. Upon treatment with the compound AP20187, iCasp9 is activated and triggers apoptosis. Interestingly, in this system, secondary cone degenerescence was not reported and rod photoreceptors did not regenerate following ablation [67]. Such discrepancies remain unexplained so far. Are cell death mechanisms induced by the

NTR/Mtz or iCasp9 systems different enough to trigger regeneration in one case and not in the other one? In line with this idea, Lee et al. observed in their rod-ablated retina that only additional injury allows for production of new rods and that it is locally restricted to the site of trauma. Thus, traumatic destruction of the retina seems required to release signals eliciting regeneration [67]. This concept nicely fits with the situation recently described in the mammalian brain where only invasive injury, such as stab wounding, leads reactive glia to acquire stem cell properties. In contrast, non-invasive brain injury, such as induced neuronal death, fails to activate proliferation of reactive glia and their conversion into stem-like cells [94]. In this model, the Hedgehog pathway was shown to be necessary and sufficient to elicit a stem cell response. Whether in the *Xenopus* retina Hedgehog is also a traumatic cue eliciting regeneration would be interesting to investigate.

4.6 Conclusions

Retinal regeneration has been extensively studied in amphibians given their remarkable ability to regenerate spontaneously. However, the molecular mechanisms underlying the different steps of the regenerative process (schematised in Fig. 4.4) still remain poorly understood and may drastically depend on the type and extent of the lesion. It is nevertheless expected that the multiplicity of experimental paradigms, including novel ones based on transgenic *Xenopus* animals, will contribute to dig further into the cellular and molecular basis of both retinal degeneration and associated regeneration. This is of particular importance to develop therapeutic strategies to treat retinal degenerative diseases. These past few years, several sources of dormant stem-like cells have been identified in the mammalian eye [72, 77]. A promising approach to replace dead neurons, alternative to tissue transplantation, could thus consist in their endogenous mobilisation. The knowledge gathered in a species able to efficiently regenerate should help to understand why regeneration is constrained in mammals and learn how to boost the naturally limited proliferative and neurogenic potential of mammalian stem-like cells.

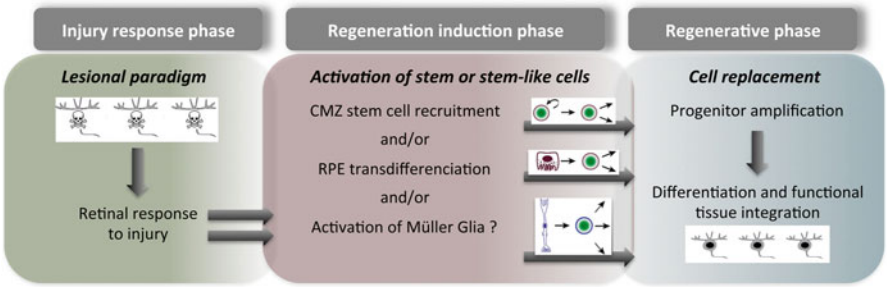

Fig. 4.4 Schematic representation of the different steps of the regenerative process in the *Xenopus* retina. Adapted from [95]

Acknowledgments The authors are supported by grants from l'Agence Nationale de la Recherche (ANR), Centre National de la Recherche Scientifique (CNRS), Université Paris-Sud, Retina France, and the Ville de Paris Région Ile de France Medicen through the AMBRe consortium. M.H. was supported by a fellowship from ANR and Région Ile de France.

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Chapter 5
Advances in Pluripotent and Adult Stem Cells for Eye Research

Gary S.L. Peh and Raymond C.B. Wong

Contents

Abbreviations

ALS	Amyotrophic lateral sclerosis
CEnC	Corneal endothelial cell
CEpSC	Corneal epithelial stem cell
CSSC	Corneal stromal stem cell
ESC	Embryonic stem cell
FD	Familial dysautonomia
iPSC	Induced pluripotent stem cell
LESC	Limbal epithelial stem cell

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A. Pébay (ed.), *Regenerative Biology of the Eye*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4939-0787-8_5,

miRNA	Micro RNA
MSC	Mesenchymal stem cell
NuRD	Nucleosome remodelling and deacetylase
SP	Side population
SSEA4	Stage-specific embryonic antigen-4
TDP-43	Tar DNA binding protein-43
TM-MSC	Trabecular meshwork mesenchymal stem cell

5.1 Introduction

In recent years, significant advances have been made in using stem cells for eye research. Conventionally, a stem cell is defined as a cell with the ability to self-renew and produce two identical daughter cells, each with the same capacity to self-renew, as well as to differentiate and commit to a specific cell lineage given the appropriate cue to differentiation [1]. Generally, stem cells can be categorized into two types, pluripotent stem cells and multipotent adult stem cells. Pluripotent stem cells have the capacity to differentiate into cells of the three embryonic germ layers: ectoderm, mesoderm and endoderm, each with the potential to further differentiate down its specific lineage into more specialized somatic cell types [2, 3]. In contrast, multipotent somatic or adult stem cells exist within various adult tissues, including haematopoietic stem cells [4], neural stem cells [5] and mesenchymal stem cells (MSCs) [6]. Furthermore, other MSCs and MSC-like cells derived from adipose tissue [7], umbilical cord [8], skeletal muscle [9] as well as tissue-specific stem cells residing within niches found within different adult tissue types such as the epidermis [10], gut epithelium [11] and corneal limbal stem cells [12] have been described. Compared to pluripotent stem cells, most of these adult stem cells are more restricted in terms of their capacity to differentiate. For instance, clonogenic plastic adherent adult MSCs isolated from bone marrow stroma, first described by Friedenstein and colleagues, can be induced to undergo adipogenic, chondrogenic and osteogenic differentiation under the appropriate conditions [13, 14].

In this chapter, we provide an introduction to pluripotent stem cells and highlight some successful examples for their uses in disease modelling and drug discovery. Also, we discuss the various populations of adult stem cells within the cornea and highlight their potentials for eye research.

5.2 Pluripotent Stem Cells

Multiple pluripotent cell types have been identified in human, including embryonic germ cells derived from foetal gonads [15], embryonal carcinoma cells derived from teratocarcinoma [16], embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Human ESCs are derived from the inner cell mass of in vitro fertilized embryos [2, 3]. These cells are pluripotent and can be propagate indefinitely while maintaining a normal karyotype. These two characteristics of human ESCs render

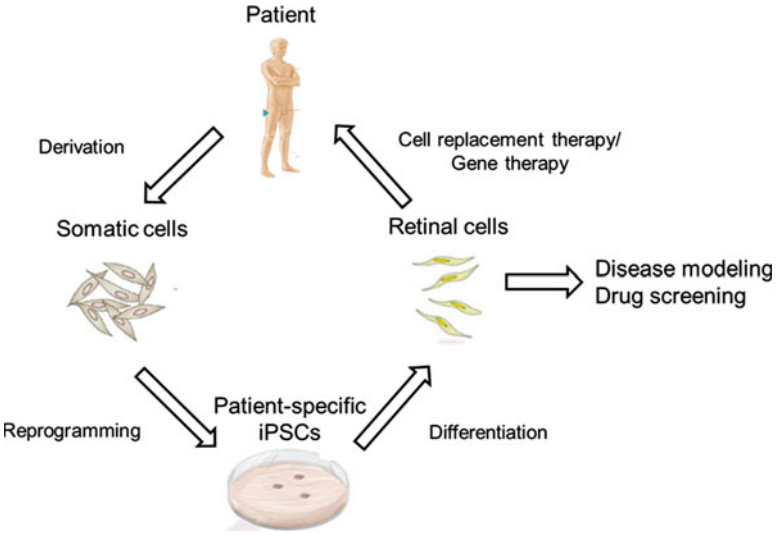

Fig. 5.1 Derivation of patient-specific iPSCs has the potentials for disease modelling and development for drug screening, gene therapy and cell replacement therapy

them an attractive cell source for regenerative medicine. However, the use of human ESCs in research has been widely debated with ethical concerns surrounding the use of human embryos for derivation of ESCs. This leads to development of method to derive human ESCs from single blastomeres that could be biopsied without destroying embryos [17]. However, such derivation method is highly inefficient and is of limited use for generation of patient-specific pluripotent stem cells.

In 2006, the seminal discovery by Shinya Yamanaka's group to reprogram adult mouse cells to iPSCs initiated a new era of regenerative medicine [18]. Shortly after, human iPSCs were successfully derived in 2007 [19, 20]. The significance of this discovery was recently recognized by the award of a Nobel Prize to Shinya Yamanaka in 2012. Unlike ESCs, iPSCs do not carry the ethical concerns with regards to research with embryos. iPSCs exhibit identical morphology as ESCs, expression of pluripotent markers and potentials to differentiate into cells representative of the three germ layers both in vitro and in vivo [19, 20]. Although subtle differences exist, overall iPSCs and ESCs display similar global gene expression profiles and epigenetic status [21, 22]. In the mouse, viable progenies have been generated exclusively by iPSCs using tetraploid complementation, the most stringent assay to demonstrate cellular pluripotency [23]. However, such assay is not feasible in human due to ethical concerns. Therefore, teratoma assay is widely recognized as the "gold standard" for pluripotency test in human iPSCs, where iPSCs are injected into immunodeficient mice to form teratoma consisting of cells representative of the three germ layers.

The development of iPSC technology allows for the generation of patient-specific stem cells, providing a platform for disease modelling and development of drug screening, gene therapy as well as cellular therapy (Fig. 5.1). Disease-specific iPSCs offer a unique source for studying pathological progression in the diseased cell types in vitro,

as well as drug screening to identify novel molecules that can reverse the diseased phenotypes to improve treatment options. Since these cells are of patient origin, cell replacement therapy using patient-specific iPSCs would exhibit minimal immune rejection following transplantation. Finally, gene therapy could be coupled with cell replacement therapy to correct genetic defects in cells derived from diseased iPSC prior to transplantation. Since the initial derivation of iPSCs, the field has moved forward at a swift pace. Significant progress has been made in new methods to enhance reprogramming efficiency and improve quality of iPSCs. Here we review the reprogramming strategies and factors used for generation of iPSCs and discuss the potentials for using iPSCs in disease modelling and drug screening.

5.2.1 Reprogramming Factors

The first iPSC generation was made possible by overexpression of four transcription factors in human fibroblasts, OCT4, SOX2, C-MYC and KLF4, commonly referred to as the "Yamanaka factors" [19]. Alternatively, a study from James Thomson's lab identified a different combination of factors to generate human iPSCs using OCT4, SOX2, NANOG and LIN28 [20]. These initial studies of iPSCs reported an extremely low reprogramming efficiency (<0.02 %). Moreover, although the inclusion of c-Myc enhanced reprogramming efficiency, it was reported to increase tumorigenicity of the derived iPSCs [24]. In an effort to increase the reprogramming efficiency and avoid the use of C-MYC, subsequent studies by other groups have reported a panel of reprogramming factors. These included Esrrb [25], L-MYC/N-MYC [24, 26], SALL4 [27], SV40 LT antigen and hTERT [28, 29]. Initially it was generally believed that OCT4 is an indispensable reprogramming factor; however, recent studies have demonstrated that iPSCs can be generated without OCT4 by replacement with Nr5a2/Lrh1 [30] or RARG/RARA [31]. Early embryonic genes such as the maternal transcription factor GLIS1 [32] and 2 cell-specific factor Zscan4 [33] were also demonstrated to play a key role in promoting iPSC generation. Furthermore, micro RNAs (miRNAs) were implicated to promote reprogramming, including mir-291-3p/mir-294/mir-295 [34] and the mir-302/367 cluster [35, 36]. Notably, the combination of mir-200c, mir-302 and mir-369 family miRNAs could be used to generate mouse and human iPSCs with relatively good efficiency [37].

One of the major obstacles in iPSC generation is to overcome cellular senescence. For instance, the efficiency of iPSC generation is significantly decreased when using high-passage somatic cells with short telomeres [38, 39]. On the other hand, knockdown of senescence factors like p53, $p21^{CIP1}$ or $p16^{INK4a}$ enhances iPSC reprogramming efficiency [38, 40–44]. Since p53 is a major tumour suppressor and has a widely recognized role in maintenance of genomic stability, it is not surprising that iPSCs generated from p53-null fibroblasts show increased chromosomal damage [38]. Although permanent knockout of p53 is not ideal for generating clinical grade iPSCs, transient shRNA knockdown of p53 seems to be acceptable as the derived iPSCs display normal karyotype [45]. Moreover, it was reported that

knockdown of p53 enhances reprogramming efficiency by 100-fold when used with the Yamanaka factors and UTF1 [44]. In this regards, a more detailed genomic analysis for iPSCs generated with p53 knockdown will be required to evaluate the safety of these iPSCs in clinical studies.

Another roadblock for reprogramming is that remodelling of the epigenetic states in somatic cells is required during early phase of induction to pluripotency [46]. Thus, one strategy to enhance reprogramming efficiency is to target chromatin modelling regulators. For instance, Mbd3 is a core member of the nucleosome remodelling and deacetylase (NuRD) repressor complex that functions in gene silencing by regulating 5-hydroxymethylcytosine-marked genes [47]. Mbd3 is demonstrated to play a critical role in maintenance of pluripotency in mouse ESCs. While Mbd3 knockout mouse ESCs are viable, they fail to differentiate in vivo in chimeric embryos and display incomplete gene silencing [48]. Moreover, knockdown of Mbd3 in mouse ESCs resulted in trophectoderm differentiation, suggesting that Mbd3 plays a role in repressing trophectoderm genes in the undifferentiated state [49]. A recent breakthrough was reported by Jacob Hanna's laboratory that depletion of Mbd3 in fibroblasts resulted in near 100 % reprogramming efficiency for both human and mouse iPSC generation within 7 days [50]. Notably, Mbd3 has been previously reported as a roadblock for reprogramming by Luo et al., where knockdown of Mbd3 enhanced reprogramming efficiency up to tenfold in the absence of c-Myc [51]. However, Luo et al. failed to report 100 % reprogramming efficiency by downregulating Mbd3. These contradictory results could be due to the fact that the "secondary cells" system used by Rais et al. represents a more sensitive system to measure reprogramming efficiencies, where all starting somatic cells carry the reprogramming transgenes and allow homogenous expression. Further research to deplete Mbd3 during iPSC generation using other starting cell types, such as keratinocytes or haematopoietic cells, would be important to understand the critical role of Mbd3 during reprogramming.

5.2.2 Reprogramming Strategies

Various delivery systems are developed to deliver the reprogramming factors for iPSC generation. The first generation of iPSCs utilized viral-based methods to deliver reprogramming factors. For instance, the initial derivation of mouse iPSCs is performed using retroviral-mediated introduction of Oct4, Sox2, Klf4 and c-Myc [18]. Retroviruses represent an efficient gene delivery system; however, transduction efficiency is low in slow dividing or non-dividing cells. Subsequently, lentiviruses are used to deliver reprogramming factors in one of the first derivation of human iPSCs [20]. Compared to retroviruses, lentiviruses offer the capability of high-efficiency infection in both dividing and non-dividing cells. Furthermore, early studies indicated that transient expression of reprogramming factors is sufficient to generate iPSCs and silencing of transgene is important for reprogramming [18, 19]. This leads to development of inducible viral vectors that provide temporal control of expression of reprogramming factors. Using tetracycline-inducible system,

iPSCs can be generated with temporal expression of exogenous transgenes during reprogramming [52–54]. One of the obstacles encountered in iPSC generation is that successful reprogramming is dependent on co-transduction of multiple viruses carrying individual reprogramming factors into a single cell. Often, low percentages of cells are infected by all viruses, leading to low reprogramming efficiency. To address this issue, polycistronic viral vectors that utilize the 2A self-cleaving peptide are adopted in the generation of iPSCs. Insertion of such self-cleaving peptide between transgenes allows ribosomal skipping, resulting in expression of multiple transgenes using a single promoter. This strategy is utilized successfully to generate mouse and human iPSCs using the Yamanaka factors with as few as a single integration [55–57].

On the other hand, the disadvantage of lentiviral or retroviral-mediated method is that they introduced undesirable genomic integration of foreign transgenes during reprogramming. Thus, excisable gene delivery systems are developed for iPSC generation, which allow subsequent removal of exogenous factors by cre-loxP system [58, 59] or piggyBac transposons [60, 61]. However, these excisable gene delivery systems may still leave undesirable alterations to the genome. For piggyBac system, excision of transgenes may lead to micro-deletion of the genomic DNA, whereas Cre-mediated excision of transgenes does not remove the loxP sites.

To address this problem, recent research focuses on the development of non-integration methods for reprogramming. Firstly, adenoviral vectors are used successfully to generate mouse and human iPSCs [62, 63], albeit with low reprogramming efficiencies. In comparison, Sendai viruses offer a highly efficient method to generate human iPSCs [64]. Temperature sensitive Sendai viral vectors are also developed to ensure removal of residual viruses following reprogramming [65]. However, both adenoviruses and Sendai viruses still require the tedious viral packaging step to prepare live viruses. Nowadays, one non-viral reprogramming method that is gaining popularity is the use of episomal vectors. Originally derived from the Epstein–Barr viruses, these episomal vectors can be transfected without viral packaging. The use of episomal vectors to generate human iPSCs is first described by James Thomson's group [66]. Subsequently, Shinya Yamanaka's group described a more efficient method using episomal vectors to deliver OCT4, SOX2, KLF4, L-MYC, LIN28 and shRNA for p53 [45]. The authors go on to show that addition of EBNA1, an essential factor for episomal amplification of the vector, significantly enhanced the reprogramming efficiency [67]. Moreover, polycistronic episomal vectors have been developed to express OCT4, SOX2, KLF4, C-MYC and LIN28, providing an integration-free approach for iPSC generation [68].

Other non-integrating, DNA-free reprogramming methods included RNA- and protein-based methods to deliver the reprogramming factors. Modified mRNAs have been used to generate human iPSCs successfully [69, 70]. Similarly, mouse and human iPSCs can be generated with mature double stranded miRNAs only, thus avoiding the use of vector-based gene transfer [37]. However, multiple transfections are required for prolonged expression of the reprogramming factors, as mRNAs and miRNAs are rapidly degraded in vitro. Recent development of self-replicative RNA simplified this process and only a single transfection is needed for generation of

human iPSCs [71]. Similarly, direct protein delivery has been described for iPSC generation by tagging recombinant reprogramming factors with the cell-permeable poly-arginine peptide [72, 73]. Subsequent research shows that activation of the toll-like receptor 3 pathway further enhanced the efficiency of reprogramming by cell-permeant protein delivery by promoting epigenetic remodelling [74].

Finally, many would consider development of an all-chemical reprogramming method as the "holy grail" in the reprogramming field, as small molecule-based method provides a completely transgene-free strategy for reprogramming that would be easy to use and highly controllable. A significant breakthrough is achieved recently by Hou et al., where the authors described an all-chemical reprogramming approach for generation of mouse iPSCs [75]. Using seven small molecules (DZNep, TTNPB, forskolin, valproic acid, CHIR99021, 616452, tranylcypromine), the authors are able to achieve a reprogramming efficiency of up to 0.2 %. It would be interesting to determine if this approach can be translated to human iPSC generation in the near future.

5.2.3 Using iPSCs for Disease Modelling and Drug Screening

In the past few years, the number of studies on iPSC application has steadily increased. Here we highlight some successful studies using iPSCs for modelling neurological diseases and drug screening.

Familial dysautonomia (FD) is a rare debilitating genetic disorder with high rate of mortality. This disorder is caused by a single point mutation of the IKBKAP gene, leading to degeneration of sensory and autonomic neurons. In 2009, Lee et al. reported the successful derivation of iPSCs from FD patients and subsequent differentiation into peripheral neurons. The authors demonstrated several phenotypes in FD-iPSCs that are relevant to the disease, including aberrant splicing of IKBKAP, defects in neurogenic differentiation and migration [76]. Further study identified the plant hormone kinetin as an effective drug to alleviate some diseased phenotypes in these FD-specific cells, including reduction of levels of mutant IKBKAP spliced form and increases in neuronal differentiation. Subsequently using high-content drug screening, the same group identified eight novel small molecules that could also rescue expression of IKBKAP [77]. Another neural disorder that was successfully modelled by iPSCs is amyotrophic lateral sclerosis (ALS). ALS is a motor neuron degenerative disorder characterized by cytosolic aggregation of Tar DNA binding protein-43 (TDP-43), resulting in paralysis and death. ALS-iPSCs have been generated by several groups [78–81]. Interestingly, motor neurons derived from ALS-iPSCs show higher level of mutant TDP-43 aggregates and recapitulated key biochemical aspects in the disease [78, 79, 81]. Moreover, these ALS-specific motor neurons display cytosolic aggregates and shorter neuritis [79]. Subsequent drug screening assays have identified novel molecules that inhibited TDP-43 aggregation and/or rescued the abnormal neuronal phenotype, including the FDA-approved drug Digoxin [79, 81]. Together, these studies demonstrated the potential of iPSCs for disease modelling and drug discovery for potential therapeutic intervention.

5.3 Adult Stem Cells Within the Cornea

The human cornea is vital for the transmission of visible light to the retina for sight perception. It is also a protective barrier, shielding the delicate internal intraocular structures of the eye from external factors or damages [82]. This unique transparent tissue is approximately 500 μm thick and is structurally organized into five distinct layers. The outermost stratified squamous, non-keratinized epithelium consisting of 5–7 rows of cells extends across the cornea surface. A narrow zone known as the limbal epithelium surrounds the border of the cornea, separating it from the ocular conjunctiva. This multi-cellular layered corneal epithelium also acts as the main protective barrier of the cornea against external environments such as UV exposure and bacterial infection [83]. The second layer that forms the outer boundary of the stroma is the acellular Bowman's membrane. Composed of various types of randomly interwoven collagen fibrils, this transparent layer is between 6 and 14 μm thick [84, 85]. The third layer is the corneal stroma, which makes up approximately 90 % of the corneal thickness. It is a densely interlaced connective tissue composed primarily of tightly aligned parallel bundles of collagen type I and IV fibrils, as well as proteoglycans [86, 87]. Residing within the organized layers of collagen lamellae are cellular network of sparsely spaced keratocytes, inter-connected with one another through distinct dendritic processes [88]. The fourth layer is the thin Descemet's membrane, which forms the inner boundary of the stroma. It is also the basal lamina of the corneal endothelium, which contributes to the overall thickness of the Descenet's membrane as an individual ages [89]. The fifth and innermost singular layer of the cornea, the corneal endothelium, plays a critical role in keeping the corneal transparent through the regulation of corneal hydration [90–92].

Diseases of the cornea leading to corneal blindness are reversible, and corneal transplantation is a viable option to restore vision once corneal clarity deteriorates. In fact, corneas are the most transplanted tissues in the world compared to solid-organ transplantations [93]. However, the numbers of corneal transplantations carried out yearly are greatly restricted by the shortage of donor corneas that are available for transplants, which remains a global issue [94]. Hence there remains a need to develop alternative treatment strategies using stem cells found within the eye.

5.4 Corneal Epithelial Stem Cells/Limbal Epithelial Stem Cells

Stem cells of the corneal epithelial layer are important for the maintenance and replenishment of the surface corneal epithelial cells throughout life. However, the identity and the exact location of the corneal epithelial stem cells (CEpSCs) have been a topic of great discussion. The most widely accepted notion of such a stem cell population is believed to be located within the limbal region of the cornea [95, 96]. However, it has also been proposed that oligopotent stem cells can be found not only in the limbal region, but also throughout the corneal epithelia [97].

It is believed that mitotically quiescent CEpSCs, termed as limbal epithelial stem cells (LESCs), are found scattered within the limbal basal layer and limbal crypts, and are known to express C/EBPδ, BMI1 and ΔNP63α—a particular isoform of transcription factor p63 [98–100]. Although these LESCs have tremendous capacity to proliferate, they are actually slow-cycling in nature [101]. When LESCs become activated in response to a wound, the ΔNP63α positive stem cells undergo asymmetric division where one of the daughter cell differentiates into a population of cells with higher proliferative capacity known as transient amplifying cells and migrates towards the central cornea. These transient amplifying cells progressively lose ΔNP63α, C/EBPδ and BMI1 expression and gain expression of ΔNP63β and ΔNP63γ, which is believed to be involved in the regulation of stratification during corneal epithelial regeneration [102]. It should be noted that various reports have also described the use of other cellular markers in the characterization of LESCs, such as the intermediate filament protein cytokeratin 15 [103], ATP-binding cassette transporter protein ABCG2 [104] and low affinity nerve growth factor receptor p75 [105]. However, most of these markers are believed to identify not only the LESCs, but also early proliferative transient amplifying cells. Taken together, these reports suggest that the co-expression of ΔNP63α, together with C/EBPδ and BMI1 may be a good indication of true LESCs with the ability to self-renew and the capacity to differentiate into mature corneal epithelial cells for the regeneration of damaged corneal epithelium. Certainly, these LESCs do not express mature corneal epithelial markers such as cytokeratin 3, cytokeratin 12, connexin 43 [106, 107] or stage-specific embryonic antigen-4 (SSEA4) [108]. In the latter case, although SSEA4 is more commonly known as a marker for human pluripotent stem cells [109], expression of this glycoprotein is found on mature corneal epithelial cells and not on LESCs, making it a potential candidate for negative selection markers for enrichment of LESCs.

The use of LESCs in clinical settings has been described for patients suffering from limbal stem cell deficiency for ocular surface regeneration as early as the late 1990s [110, 111]. Significant improvements of the in vitro expansion of LESCs using a feeder-free explant culture approach and xeno-free products have been described [112]. However, the standard expansion of LESCs grown on clinical-grade 3T3-J2 mouse feeder cells is acceptable for current clinical practises, as the key determining factor of a successful LESC-transplantation outcome is the amount of p63-bright cells, which in turn lead to the regeneration of the corneal epithelium [113].

5.4.1 Corneal Stromal Stem Cells

The corneal stroma makes up the majority (approximately 90 %) of the corneal thickness, contributing to both the strength and transparency of the cornea. Residing within the uniformly organized collagen fibril and interfibrillar spacing of the stroma are stromal keratocytes, responsible for synthesizing and secreting keratin sulphate proteoglycans such as lumican, keratocan, mimecan and decorin [87, 114]. These proteoglycans are required for the development of organized collagenous

matrix, which is essential for corneal transparency [115]. Most of the stem cell research in cornea to date has been primarily focused on LESCs. However, over the last decade, various studies have sought to delineate factors that may be involved in the regulation and renewal of the corneal stroma keratocytes, and that the stromal layer may also contain a population of adult stem and progenitor cells. Indeed, approximately 3 % of isolated adult bovine stromal cells were shown to have progenitor-like characteristics with the capacity for clonal growth and over 50 population doublings, without losing the potential to form keratocytes that are positive for the markers keratin sulphate, keratocan and ALDH3A1 [116].

The ability to efflux fluorescent dye such as Hoechst 33342, first described in haematopoietic stem cells [117], has been used as a method to identify and isolate populations of putative stem and progenitor cells termed the "side population" (SP). This SP fraction identifies a variety of cell types such as cardiac [118], myogenic [119] and dental pulp cells [120], as well as from ESCs [121]. Interestingly, the SP cell fraction makes up less than 1 % of isolated corneal stroma cells. These SP cells are known as corneal stromal stem cells (CSSCs) and can be expanded over 100 population doublings [122], far superior than the progenitors of keratocytes described earlier. CSSCs were also shown to display MSC-like property and expresses stem cell markers such as ABCG2, BMI1, CD166, cKIT, PAX6, SIX2 and NOTCH1 [122]. It is believed that these CSSCs reside in the limbal stroma, subjacent to the basement membrane of the LESCs [116]. However, no study has demonstrated in vivo multipotentiality of isolated corneal keratocytes until recently. When human foetal keratocytes were isolated and injected into embryonic chick, they were able to respond to embryonic cues and differentiate into various neural crest derivatives such as smooth muscle in cranial blood vessels, stromal keratocytes and corneal endothelium [123]. It will be interesting to see if CSSCs isolated from adult stroma possess similar differentiation potential as these primitive human foetal keratocytes, as it can form the basis of isolating CSSCs for regenerative medicine for stroma keratocytes and corneal endothelium.

5.4.2 Corneal Endothelial Stem Cells

The mono-layered corneal endothelial cells (CEnCs) are made up of mostly cells that are hexagonal in shape, but five- to eight-sided cells have also been reported [124]. These CEnCs form a distinctly tight endothelial mosaic and is believed to communicate intercellularly through gap junctions via the extensive inter-digitations found between their lateral membranes [125]. Also, tight junctions complexes located at the cellular perimeter of the CEnCs form a "leaky" cellular barrier, which is important for the passive permeation of nutrients from the aqueous humor into the stroma layer that sustains the stroma keratocytes [90, 126]. This continuous influx of solute and fluid is actively moved out of the stroma back into the aqueous humor via metabolically active pumps operating throughout the corneal endothelium [92, 127]. This dynamic barrier and pump function of the corneal endothelium is

critical in the regulation of corneal hydration, as well as preservation of corneal transparency.

It is generally agreed that the CEnCs do not undergo any functional regeneration within the eye [128, 129]. Hence, when CEnCs are damaged, adjacent cells spread out to maintain an intact cellular layer in order to preserve the delicate functional integrity of the corneal endothelium [130]. The occurrence of cell spreading can be associated with an increase in both pleomorphism and polymegathism of CEnCs seen in older individuals [131]. When an acute loss of CEnCs occurs, either due to accidents, surgical traumas or the onset of corneal endothelial diseases, decompensation of the cornea will occur to a degree that affects the functional capacity of the corneal endothelium [94]. Deterioration of visual acuity will follow as the stroma becomes oedematous, which will eventuate to corneal blindness. Restoration of vision is possible by replacing the dysfunction corneal endothelium with a healthy donor corneal endothelium through corneal transplantation [93]. This is a one-to-one donor-to-recipient surgery, which is severely hindered by the global shortage of donor graft material that is available for transplants [94].

Although the CEnCs do not replicate within the eyes, these cells can be expanded in vitro under the appropriate culture conditions [94, 132]. As such, it has enabled the development of potential alternate treatment strategies via cell-tissue engineering [133–135] or cell-injection therapy [136] to alleviate the dependence of donor graft material. However, the proliferative capacity of the primary CEnCs is considerably limited, especially when compared to the expansion capacity of a true self-renewing stem/progenitor cell populations discussed earlier. Furthermore, initiation and establishment of growing cultures of primary CEnCs still requires the use of donor corneal tissues.

As mentioned earlier, the clinical use of LESCs has been established since the late 1990s. However, no stem cell therapy has been developed for the corneal endothelium, as a true stem cell of the corneal endothelium remains elusive. Studies have proposed that stem cells of the corneal endothelium reside within a region between the very edge of the corneal endothelium and the trabecular meshwork [137]. These peripheral CEnCs have been shown to express Lgr5, a marker that identifies stem cells of the intestine, colon, stomach and hair follicle in mice [138–140]. McGowan and colleagues detected the expression of ESC marker OCT4 and early neural markers SOX2 and PAX6 in the peripheral region of corneas, “wounded” from the removal of the central cornea for transplantation by trephination [141]. Although these studies suggested that the peripheral area of the corneal endothelium expressed certain embryonic and adult stem cells markers, it did not show any conclusive evidence to satisfy some of the key criteria of a stem cell, such as the ability for clonogenic self-renewal or the potential to commit into a differentiated progeny.

A region of interest, the trabecular meshwork, lies just beyond the edge of the corneal endothelium and the transition zone. The trabecular meshwork is a meshwork of porous tissue, and several studies have successfully isolated primary cells from trabecular meshwork [142–144], which can be grown as a spheroid culture [145]. However, it is not until recently that these trabecular meshwork cells, termed here as TM-MSCs, were shown to possess MSC-like properties [146].

These TM-MSCs express typical MSC markers such as CD73, CD90 and CD105; form adherent colonies and can be differentiated into osteocytes, chondrocytes and adipocytes when exposed to the appropriate culture conditions [146]. Due to the close proximity of the trabecular meshwork to the CEnCs, it is plausible to think that these TM-MSCs may possess the capacity to differentiate into CEnCs as well; however, further studies will be required to ascertain this theory.

Using an alternative approach, Hatou and colleagues showed that progenitors of the corneal stroma could be differentiated to form corneal endothelial-like cells through the modulation of retinoic acid and Wnt/β-catenin signalling [147]. As both the corneal stroma and corneal endothelium are of neural crest origins [148], it may be possible to direct the differentiation of CEnCs using human ESCs or iPSCs by first inducing the formation of neural crest cells, which has been described [149, 150]. Though still in its infancy, such an approach using pluripotent stem cells holds great potential in the differentiation of putative CEnCs for the development of alternative corneal endothelium replacement strategies, as it can truly eliminate the need for donor corneas altogether.

5.5 Summary and Future Directions

Although still at an early stage, pluripotent and adult stem cells show great potentials in eye research. For pluripotent stem cells, advances in reprogramming methods have improved the efficiency and the quality of the derived iPSCs, with minimal genetic modification and lowered risks of tumorigenicity. In addition, iPSCs have also proved to be an invaluable resource for disease modelling and drug discovery for several neurological diseases. In recent years, studies using iPSCs for modelling ocular disease are gaining momentum, including retinitis pigmentosa [151, 152], Best disease [153] and glaucoma [154]. Future research using iPSCs to model ocular diseases will prove helpful to understand the mechanisms responsible for disease progression and potentially identify novel drug treatments.

On the other hand, the use of endogenous stem cell population within the eye to repair damages is an attractive strategy to combat ocular diseases. To date, this has been well established for the use of LESCs within a clinical setting for some diseases of the ocular surface. However, other adult stem cell populations purportedly described to be found within the cornea require more extensive studies and characterization before their translational values can be realized. For example, human CSSCs injected into Lumican-null mice with corneal opacity due to disruption of stromal collagen organization were able to restore matrix organization and corneal transparency [155]. Whether such an approach is applicable for the treatment of human corneal diseases with similar types of corneal opacities remains to be established. Further, the identity and exact location of a “true” corneal endothelial stem or progenitor cells that satisfy the basic criteria of a stem cell still remains elusive. Though foetal keratocytes have the capacity to form neural-crest derivatives including corneal endothelium, it remains to be seen if these primitive keratocytes,

TM-MSC, or iPSCs can be differentiated to form functional CEnCs within an in vitro setting. Nevertheless, we must appreciate the collective efforts in the search for potential alternative treatment strategies using these endogenous stem cells, as it opens up exciting prospects for ocular cell and regenerative medicine.

Acknowledgements The authors are grateful for support from National Research Foundation Translational and Clinical Research Programme Grant (R1020/35/2013TCR) (G.S.L. Peh), Biomedical Research Council Translational Clinical Research Partnership Grant (R827/22/2011TCRPA) (G.S.L. Peh), Cranbourne Foundation (R.C.B. Wong), ORIA/RANZCO Eye Foundation Grant (R.C.B. Wong) and the University of Melbourne ECR Grant (R.C.B. Wong). The Centre for Eye Research Australia (R.C.B. Wong) receives operational infrastructure support from the Victorian government.

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Chapter 6
Stem Cell Strategies for Optic Nerve Protection

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Contents

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A. Pébay (ed.), *Regenerative Biology of the Eye*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4939-0787-8_6, © Springer Science+Business Media New York 2014

Abbreviations

AAA	Alpha aminoadipic acid
AD	Alzheimer disease
BDNF	Brain neurotrophic factor
CMZ	Ciliary marginal zone
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CRVO	Central retinal vein occlusion
CSPG	Chondroitin sulfate proteoglycans
bFGF	Basic fibroblast growth factor
ES	Embryonic stem cells
GDNF	Glial cell-derived neurotrophic factor
GFAP	Glial fibrillary acid protein
IOP	Intraocular pressure
IPS	Induced pluripotent stem cells
LGN	Lateral geniculate nucleus
MAG	Myelin-associated glycoprotein
MSC	Mesenchymal stem cells
MS	Multiple sclerosis
NPC	Neural precursor cells
ON	Optic nerve
ONH	Optic nerve head
OEC	Olfactory ensheathing cells
OPC	Oligodendrocyte precursor cells
PD	Parkinson's disease
PNS	Peripheral nervous system
RGC	Retinal ganglion cell
RGCL	Retinal ganglion cell layer
SAPNS	Self-assembling peptide nanofiber scaffold
SGZ	Subgranular zone
SVZ	Subventricular zone
VEGF	Vascular endothelial growth factor

6.1 The Eye as a Window to the Brain

The eye develops initially as an extension of the diencephalon and the optic nerve and retina are considered part of the central nervous system (CNS). The eye shares many anatomical, functional and immunological features with the brain and the spinal cord. As elsewhere in the CNS, complex neuronal circuits in the retina process information and connect to other centres in the brain.

The way the retina and the optic nerve respond to insults is also similar to other parts of the brain. As in the brain and the spinal cord, an insult to the optic nerve may

result in neuronal loss and the creation of a hostile and neurotoxic environment that inhibits regeneration and may lead to the death of neighbouring neurons [1–3].

Moreover, several neurodegenerative conditions affecting the brain and the spinal cord have manifestations in the eye and ocular symptoms may precede the diagnosis of such CNS disorders [4–8]. In multiple sclerosis, for example, optic neuritis associated with demyelination and RGC degeneration is diagnosed in 75 % of patients and is often a presenting feature [4].

In addition, several eye-specific diseases share features with other CNS pathologies [9–11]. In glaucoma, for instance, RGC body loss is associated with axonal atrophy, deficits in axonal transport and deposition of amyloid β and p-tau, as in Parkinson's disease and Alzheimer's disease [11, 12].

These similarities and parallels have led researchers to consider the eye as a valuable and relatively accessible model to study the CNS in health and disease. Additionally, examination of the eye is often useful in the diagnosis of CNS disorders. Thus, researchers and clinicians often consider the eye as a window to the brain.

6.1.1 Key Players in Light Perception: The Retina and the Optic Nerve

The sense of sight is the result of the interaction of light, eyes and brain. Light entering the eye generates nervous signals which are sent to the brain. Nervous impulses are deciphered in the brain and images are perceived. This succession of events begins in the retina and in the optic nerve, key components of the visual pathway.

The retina, light-sensitive tissue lining the inside of the eye, is where the detection and signalling of light occurs. The retina is characterised by several layers of neurons interconnected by synapses in the plexiform layers. When light hits the retina, the first cells to respond are the photoreceptors, located in the outermost part of the retina. A cascade of chemical and electric events occurs in photoreceptors resulting in a change in membrane potential and modulation of neurotransmitter release at their synapse with bipolar cells. Nervous impulses, modulated by interneurons (bipolar, horizontal and amacrine cells) in the inner nuclear layer, are sent to the retinal ganglion cell layer (RGCL) in the innermost part of the retina. Here, retinal ganglion cells (RGCs) respond by firing action potentials along their axons to target areas in the CNS. RGC axons bundle together at the optic nerve head (ONH), where they take a 90° turn away from the eye and proceed to the brain in the optic nerve.

The optic nerve, as a cable of nerve fibres, carries information from the eye to the brain.

Characterized by myelinated fibres and ensheathed in all three meningeal layers, the optic nerve runs from the eye towards the optic chiasm in the brain, where decussation occurs. From this point, most axons are directed to the lateral geniculate nucleus (LGN), from where post-synaptic neurons project to the visual cortex. Other fibres terminate in the superior colliculus, in the pretectal nucleus and in the suprachiasmatic nucleus, respectively involved in voluntary eye movements, reflex eye movements and the sleep–wake cycle.

Given the critical role of the optic nerve in transferring visual information, optic nerve disorders frequently reduce vision and can affect one or both eyes.

6.2 Diseases of the Optic Nerve

A variety of disorders can insult RGCs and the optic nerve, including neurodegenerative, ischemic, traumatic and inflammatory diseases [10, 13, 14]. The most common optic nerve diseases include glaucoma, ischemia, trauma and inflammatory optic neuropathy [15]. Although the underlying cause might vary, in many cases these pathological conditions result in serious visual impairment due to the progressive RGC loss and optic nerve degeneration [16].

6.2.1 Glaucoma

Glaucoma is a chronic, degenerative optic neuropathy that remains the leading cause of irreversible blindness worldwide [17, 18]. Characterized by the selective loss of RGCs and optic nerve (ON) damage [19], glaucoma is sometimes known as "the silent thief of sight", due to its painless and asymptomatic onset. Indeed, the diagnosis is often delayed and glaucoma is frequently undetected until significant optic nerve damage has already occurred [20, 21].

Over the last few decades, several risk factors have been associated with glaucoma onset, including age, race and genetic factors [21, 22]. However, the key modifiable risk factor is elevation of the intraocular pressure (IOP) [23, 24], resulting from the altered drainage of the aqueous humour. Elevation of eye pressure is a risk factor for progressive damage to the optic nerve and gradual loss of RGCs [19, 25, 26].

Although glaucoma is not always associated with IOP elevation [27], most animal models of the disease involve raised eye pressure. Glaucoma models have been very useful to understand many processes underlying RGC death [28]. The relationship between axonal injury and RGC loss in glaucoma is incompletely understood. It seems likely that biomechanical deformation of the ONH increases the risk of axonal atrophy and subsequent RGC death, and ischaemic mechanisms may also be important [29–31]. The balance between different mechanisms may well vary in different individuals and between different types of glaucoma.

To date, lowering the eye pressure is the only proven treatment for glaucoma that reduces the risk of further deterioration. Nevertheless, many patients continue to deteriorate even when a low eye pressure is achieved [32], suggesting that RGC death and optic nerve degeneration may occur via different mechanisms. Therefore, new scientific and clinical approaches are needed.

6.2.2 Ischemic Optic Neuropathy

Retinal and optic nerve ischemia, common causes of visual impairment in the middle age and elderly population [13], occur when the tissue blood supply is reduced to an insufficient level.

Ischemia is a consequence of local circulatory failure affecting the venous or arterial side of the circulation. When retinal or optic nerve ischemia occurs, the tissue, deprived of oxygen, nutrients and of a way to dispose cellular waste, undergoes energy deprivation and subsequent cellular death. Several animal models have been used to understand the mechanisms involved in neuronal loss after ischemia of the retina and optic nerve. RGC death is associated with a cascade of destructive events, initiated by mitochondrial dysfunction and followed by neuronal depolarization, calcium influx, oxidative stress and subsequent cell death [33]. Reactive gliosis is also likely to contribute to RGC loss [34]. To date, several treatments have been found to attenuate RGC death and axonal damage in experimental model of retinal ischemia, including anti-inflammatory agents, neurotrophic factors and glutamate antagonists [34, 35]. However, none of these approaches have yet been proven to reduce disease glaucoma onset or progression in human glaucoma.

6.2.3 Traumatic Optic Neuropathy

Traumatic optic neuropathy refers to an acute injury to the optic nerve often resulting in transection of RGC axons leading to partial or total loss of vision. Axotomized RGCs undergo apoptotic cell death following the injury. The rate of neuronal death generally depends on the site of injury. According to studies performed on animal models of optic nerve crush, the closer the injury is to the ONH, the quicker the RGC loss. Given the inability of neurons to regenerate axons, research has been mainly focused on neuroprotective and regenerative strategies in order to attenuate the inevitable death of RGC and promote axonal regrowth.

6.2.4 Inflammation and Other Neurodegenerative Conditions

As an extension of the CNS, the eye also suffers the consequences of other inflammatory and neurodegenerative conditions primarily affecting the brain and the spinal cord. Indeed, as already mentioned, RGC death and axonal degeneration may be observed in disorders such as Multiple Sclerosis, Alzheimer disease, Parkinson's disease and stroke [4–8].

Optic neuritis is the most common inflammatory condition affecting the optic nerve. Associated with RGC loss and demyelination of axons along the visual pathway, optic neuritis is often an early feature of MS [4]. Inflammatory cues together with myelin debris have been proposed as major factors responsible for axonal injury and regenerative failure [36].

Impaired visual acuity has also been described in PD [6], where optic nerve degeneration may also occur, although the magnitude and mechanism of such an effect remains uncertain [6, 37].

Alzheimer's disease has also manifestations in the eye, where amyloid β and p-tau accumulate in the retina [38] and are associated with loss of RGCs and axonal degeneration [39].

6.3 Retinal Ganglion Cell Death and Optic Nerve Degeneration

Optic neuropathies involve RGC death and optic nerve degeneration. Which of these two events happens first depends on the disease [4, 6, 33] and in some of the cases, such as in glaucoma, is still not clear [19]. Optic neuropathies lead to visual deficits that are usually irreversible because of the inability of neuronal cells to regenerate axons. With the ambitious goal of reversing visual loss, recent research in regenerative medicine has focused on the biology of RGCs in order to better understand the mechanisms underlying RGC death and neuronal regenerative failure.

6.3.1 RGC Death

Apoptosis and necrosis are important mechanisms of neuronal death. Apoptosis involves a series of controlled biochemical events, such as nuclear and DNA fragmentation, chromatin condensation and cell shrinkage, leading to programmed and orderly cell death. In necrosis, cell death is less ordered and necrosis is frequently associated with acute and chronic inflammation. In most optic neuropathies, apoptosis is a major mechanism of RGC death [34, 40]. Depending on the disorder, the insult can primarily affect the RGC body or its axon [15]. Organelles of RGCs, including the mitochondria, endoplasmic reticulum, Golgi and cytoplasm, are generated in the cell body and are transported along axons in order to maintain the cellular environment [41]. In the same way, RGC axons supply the cell body with trophic factors retrogradely transported in microsomal vesicles [42]. When an insult occurs, this equilibrium between axon and cell body fails and cell death is triggered [33, 43]. Axons and soma may die via distinct mechanisms [35, 44], with cell body loss usually occurs by apoptosis [40]. This phenomenon is known as primary degeneration of the optic nerve. Reactive gliosis, excitotoxicity, oxidative stress, hypoxia and inflammation may also occur and spread the damage beyond the initial site of injury leading to the death of neighbouring neurons [45, 46]. This phenomenon is known as secondary degeneration. Similar to other CNS diseases, secondary degeneration may determine the final extent of impairment and may continue even after termination of the primary insult. In glaucoma, for instance, the process of RGC death seems to be initiated at the ONH. Here, often as a result of IOP elevation, a combination of events thought to include mechanical compression and ischemia [29, 47], together with reduced neurotrophic support [30, 48], β-amyloid deposition [49], oxidative stress [50] and possibly excitotoxicity [51, 52], trigger the apoptotic cascade. Evidence that RGCs continue to deteriorate even when a low eye pressure is clinically achieved [53] suggests that mechanisms of secondary degeneration may also be involved. Although the role of secondary degeneration in optic nerve diseases remains incompletely understood, an experimental model has recently been developed [2], allowing morphologic separation between primary and secondary degeneration. Studies using this model have demonstrated that apoptotic RGC death may also take place remote from the site of injury [46].

6.3.2 Failure of Optic Nerve to Regenerate

As in other parts of the CNS, failure of the optic nerve to regenerate remains a main challenge to overcome in neurodegenerative diseases and after injury. In recent decades, regenerative medicine approaches have been extensively focused on trying to achieve a better understanding of what limits regrowth of RGC axons, with the final aim to identify potential treatments able to slow down or reverse visual loss. The evidence that axons in the mature CNS cannot renew themselves after injury as occurs in the peripheral nervous system (PNS) [54] and that the neonatal CNS retains its ability to grow axons till a certain developmental stage [55] suggests that extrinsic and intrinsic factors are involved in the failure of adult neurons to regenerate. Although further investigation is still needed, progress has been made in understanding the cellular and extracellular inhibitory environment after injury. Over the last two decades, important molecules and pathways either facilitating or limiting axon regeneration have been identified and a clearer picture of the relevant mechanisms has emerged.

6.3.2.1 Extrinsic Inhibitory Factors

The lack of neurotrophins, such as brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF) and basic fibroblast grow factor (bFGF), together with the inhibitory environment of the optic nerve at the site of injury, are considered major extrinsic mechanisms contributing to RGC death and regeneration failure.

Lack of Neurotrophic Support

Neurotrophins are a family of proteins contributing to survival [56] and function of neurons [57]. They have also been seen to play an important role during development, when RGCs produced in excess die after failing to contact their central target [58]. Moreover, another study has shown that RGCs died after their target neurons in the LGN were eliminated by kainic acid and described the neurotrophins dependence among RGCs [59]. When an insult occurs, the connections of RGCs to their targets are impaired or, in the worst cases, completely disrupted. This results in the loss of target-derived neurotrophic support to RGCs [30]. In glaucoma, for instance, IOP-induced mechanical stress and hypoxia cause axonal compression, swelling and subsequent retrograde transport obstruction at the ONH [31, 47]. In cases of traumatic optic nerve injury the situation is even more dramatic when there is partial or complete transection of the optic nerve. In this case, the closer the damage is to the ONH, the more rapidly RGCs die [43]. Animal models of experimental glaucoma and optic nerve injury have revealed that exogenous application of BDNF or CNTF promotes RGC survival after elevated IOP [48] or optic nerve injury [60], supporting the hypothesis that in different optic neuropathies reduced trophic support is involved in progressive RGC loss.

The Inhibitory Environment of the Optic Nerve

The lack of regenerative growth in the mature CNS after an insult has commonly been attributed mainly to the environment of the site of injury. Comparing PNS to the CNS, researchers have tried to identify what factors facilitate regeneration in the PNS and block regeneration in the CNS. One of the major differences between PNS and CNS is the local population of glial cells. In the PNS, the glial component is represented by Schwann cells which ensheath the peripheral nerve fibres and are protective and supportive of axon growth. In the CNS, the glia component includes oligodendrocytes and reactive astrocytes. Although usually protective and supportive of neurons, under pathological circumstances glia may have detrimental effects on regeneration. Oligodendrocytes are the counterpart of the Schwann cell in the CNS, myelinating, sustaining and protecting RGC axons beyond the lamina cribrosa. However, when an injury to the optic nerve occurs, oligodendrocyte degenerate leaving myelin debris at the site of injury. Myelin debris contain proteins such as NogoA, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein which are inhibitory to axonal growth. Astrocytes, on the other hand, react to the damage by undergoing reactive gliosis, a cascade of molecular, biochemical and morphological events resulting in the formation of a glial scar. In the glial scar, glial cells proliferate, become hypertrophic and secrete inhibitory extracellular matrix molecules, such as chondroitin sulfate proteoglycans (CSPGs). This process results in the generation of a physical and molecular barrier to axonal re-growth [36, 61].

6.3.2.2 Intrinsic Inability of Adult RGCs to Regenerate

Evidence that the environment at the site of injury is inhibitory to axonal re-growth [62] has allowed researchers to investigate possible ways to promote regeneration. Blocking the Rho/ROCK pathway, a downstream target of most of the inhibitory environmental signals [63, 64], or enzymatic digestion of CSPGs by using chondroitinase ABC [61, 65], may facilitate RGC axonal growth. However, only a small percentage of adult RGCs regenerate axons suggesting that removal of environmental barriers to axonal growth is not sufficient to promote significant regeneration [64]. The situation is different in the neonatal CNS, where neurons spontaneously regenerate axons after injury [66]. This observation, together with the finding that embryonic retinal explants can extend axons into adult or embryonic brain while adult retina cannot [67], strongly suggests that the environment is not the only limit to regeneration. Indeed, further studies have demonstrated that part of the problem is within RGCs themselves, which lose their intrinsic capability to re-growth axons during early development [68]. Induction of an inflammatory reaction in the eye, by puncturing the lens for instance, seems to partially overcome this limitation [69–71]. In this regard, lens injury triggers an inflammatory response involving activation of signalling pathways such as JAK/STAT3 [72] leading to the up-regulation of genes related to RGC axonal growth such as GAP-43 [64, 72]. Despite these promising findings, the adverse effects

associated with inflammation, such as oxidative stress and excitotoxicity, might still represent an obstacle to therapeutic application of such strategies [73, 74].

6.4 Stem Cell Therapy for Optic Nerve Protection

Current therapeutic approaches for optic nerve disease are limited. In glaucoma, for instance, pharmacological reduction of IOP and surgical or laser interventions to enhance aqueous circulation and drainage are the mainstay of treatment [75]. However, although these therapies succeed in slowing down the progressive loss of RGCs, their effect is often incomplete and irreversible visual loss still occurs in a significant proportion of the cases [76]. Therefore, current research aims to develop novel treatments using alternative strategies. In this regard, advances in stem biology have raised hopes that stem cell transplantation may be a potential approach for both neuroprotective and regenerative purposes. Recent studies show the practicality of protecting or replacing lost host neurons by using stem or progenitor cells [77, 78]. However, outstanding problems and unanswered questions remain to be addressed before cell therapy can be translated into the clinic. What types of cells should be transplanted? What is the best way to engraft them? What are the related obstacles and how to cope with them? Is transplantation into the eye sufficient to achieve an optimal therapeutic effect? Might stem cell therapy converge into clinical practice one day?

6.4.1 Sources of Stem Cells

By definition, stem cells are immature, uncommitted cells able to self-renew indefinitely and able to differentiate into different cell types under appropriate stimuli or environmental conditions. Traditionally, stem cells have been classified according to potency.

Pluripotent stem cells, such as embryonic stem cells (ES), are able to generate cell type of any lineage, including retinal neurons as confirmed in recent in vitro studies [79, 80]. This feature suggests great potential in regenerative medicine and ES cells are arguably the most promising source at the present time. Some concerns remain over using this class of stem cells for therapeutic purposes. The risks of tumourigenesis and rejection appear to be lower than those for other types of stem cells [81], but ethical issues related to ES cell isolation from human embryos need to be considered. Another source of cells with similar potential but less ethical concerns are induced-pluripotent stem (IPS) cells. IPS cells are cells with embryonic stem cell-like properties that can be generated from somatic cells by inducing the expression of specific genes [82]. Interestingly, iPS cells were recently induced to differentiate in vitro into RGCs that could be injected into the eye [83]. However, the risk of tumour formation from IPS-derived cells remains a concern.

Multipotent stem cells, also known as adult/somatic stem cells, are derived from discrete niches in the adult organism, where they survive and divide in order to generate new cells required for the tissue maintenance. Having lost their totipotency during development, somatic stem cells are able to generate only cells of a certain lineages under physiological conditions. However, despite the limitation of being lineage-restricted, multipotent stem cells possess many advantages. First of all, they can be harvested from patients for autologous transplantation, overcoming the risk of immunological rejection. Moreover, unlike ES cells, they provide a cell source with no ethical concerns. Somatic stem cells for retinal repair and optic nerve protection can be derived by umbilical cord and bone marrow, hippocampus or forebrain, retina and olfactory mucosa [84].

Mesenchymal stem cells (MSCs) are usually derived from umbilical cord or bone marrow. They have been demonstrated to be able to differentiate into a variety of cell types, such as adipocytes osteoblasts and chondrocytes [85]. Moreover, the evidence that they express native immature neuronal proteins [86] and that under certain in vitro experimental condition the expression of such neuronal markers can be increased [87] has led researchers to investigate the capability of this cell population to transdifferentiate into neurons in vivo. Studies so far have been challenging to interpret and the relevance of what has been seen in vitro remains to be established [88]. To date, it has proven difficult to derive functional neurons from transplanted MSCs in animal models. Nevertheless, although the potential of this class of cells to generate brain cells remains uncertain, their ability to confer optic nerve protection by their trophic and immunomodulatory properties is clear [77, 89].

Neural stem cells (NSCs) are somatic cells located in specific niches of the brain, in particular in the subventricular zone (SVZ) and subgranular zone (SGZ). Their demonstrated ability in vitro to generate cells of the CNS, such as neurons, astrocytes and oligodendrocytes, makes them a good candidate not only for neuroprotective but also for regenerative purposes. However, in vivo studies to date have shown limited evidence of successful differentiation and integration of transplanted neural precursor cells (NPCs) into the host. Indeed, despite expressing early neuronal cell markers once transplanted into the eye, NPCs only moderately integrate the retina and do not seem to mediate functional improvement [90]. On the other hand, as for MSCs, there is strong evidence of neuroprotective and immunomodulatory properties of NSCs after transplantation in the lesion site of injured optic nerve [91, 92].

Another promising source of cells highly neuroprotective to retina and optic nerve belongs to the glial lineage and consists of oligodendrocyte precursor cells (OPCs), olfactory ensheathing cells (OEC) and Muller cells.

*OPC*s are progenitors of oligodendrocytes and other cell types within the CNS. OPCs play a major role in myelinating CNS axons in order to protect and support the axons and facilitate rapid propagation of action potentials. OPCs have been reported to have some stem cell features and neuroprotective potential in vitro [93, 94]. Based on this evidence, OPCs have recently been investigated in an animal model of glaucoma. Interestingly, when transplanted intravitreally OPCs confer long-term neuroprotection, probably by the release of diffusible trophic factors [95]. Moreover, under inflammatory stimuli, they are also able to myelinate RGC axons, a potential

that may be of interest in case of demyelinating disorders such as optic neuritis in MS [96]. Similar success has been also achieved by transplanting Schwann cells, counterpart of oligodendrocytes in the PNS [97].

*OEC*s are cells of the olfactory bulb, guiding and ensheathing axons of the olfactory nerve from the nose to the brain. In models of spinal cord injury OECs successfully support axons and restore function [98, 99], leading to investigations into their potential use as cell therapy in optic neuropathies. In a model of optic nerve trauma, OECs were found not only to prolong RGC survival [100] but also to promote regeneration. Indeed, when injected in the lesion site, they migrated far from lesion and facilitated RGC axonal regeneration to the extent of their migration [101]. Of relevance for glaucoma and optic neuritis, there is evidence that, when transplanted intravitreally, OECs migrate into the ONH and ensheath unmyelinated RGC axons providing trophic and mechanical support [102].

Muller cells are retina glial cells with stem-like properties. In chicks, zebrafish and amphibians, for instance, injury induces Muller cells to undergo dedifferentiation, re-enter the cell cycle and generate neuron-like cells [103, 104]. Despite mammals having lost this regenerative potential, there is evidence that mammalian Muller glia can still self-renew and differentiate into neuronal cell types, both in vitro and when transplanted in the lesioned retina [105]. In this regard, a subpopulation of Muller cells with stem cell characteristics (MIO-M1 stem cells) has been identified in the human retina [106] and, even more interestingly, they seem able to produce cells expressing neuronal and glial markers when transplanted within the glaucomatous eye [107]. In addition, another population of cells with stem cell-like properties has been also found in the ciliary marginal zone (CMZ) of the mammalian retina, where cells are able to divide extensively and generate retinal neurons and glial species [108]. Among all the different stem cell sources being investigated so far, retinal progenitors appear particularly successful when it comes to differentiation into retina-specific cells [109, 110].

6.4.2 Transplantation Strategies

The ultimate goal of regenerative medicine for optic neuropathies is to identify optimal strategies to preserve or, more ambitiously, reverse visual loss. Much current research is focused on identifying the best source of stem cells and the best way to engraft them (Fig. 6.1).

The optimal stem cell type and the best route depend on the ultimate goal. If the final aim is neuroprotection, a good candidate is a population of stem cells able to support surviving cells, independently on their capability to differentiate into neurons or to acquire neurological functions. On the other hand, cell replacement and regeneration require a source of stem cells able to migrate, integrate into the host tissue, differentiate into a specific cell type and form functional synaptic connections to restore vision. Once a good candidate has been identified, the next step is to identify an efficient delivery mechanism. Cells can be injected intravitreally or

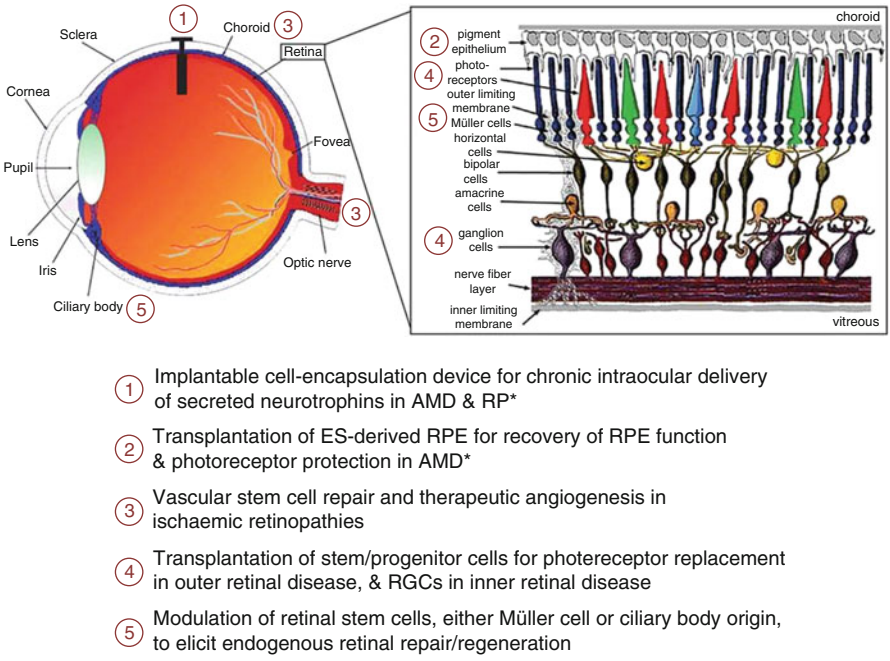

Fig. 6.1 Schematic of the human eye and retinal anatomy highlighting experimental stem cell therapies under investigation. The possibility of using stem cells for neuroprotection and/or regeneration is being explored for several common neurodegenerative conditions. Diagram courtesy of Webvison, an online resource from the John Morgan Eye Center, University of Utha, Utha. Figure modified from our published review [137]

subretinally. Alternatively, they can be injected at the ON lesion site or intravenously. So far, intravitreal transplantation and injection onto the injury site seem to be the most successful route for promoting RGC survival, replacement and regeneration. Indeed, several attempts have previously been made to deliver cells subretinally, but these approaches turned out to be unsuccessful. Indeed, cells subretinally transplanted barely get to the RGCL and cells delivered through the blood system hardly cross the blood retinal barrier.

6.4.2.1 Neuroprotection

Rescue of damaged RGCs by neuroprotective strategies has been investigated in an attempt to attenuate the inexorable and progressive loss of vision typically occurring during optic neuropathies. There are several advantages to using stem cells for neuroprotective purposes. Firstly, neuroprotective cell therapy could potentially provide long-lasting effect after a single treatment and therefore may require less frequent administration compared to many pharmacological approaches. Moreover, as observed for NPCs and MSCs, cell therapy may

facilitate neuronal survival by acting on the surroundings, for example by making the local environment more permissive [111–113].

Stem cells could conceivably be transplanted intravitreally or directly at the site of injury in the ON, depending on the disease process. The mechanisms through which neuroprotection is achieved in animal models mainly depends on the phenotype of stem cells used. Generally, secretion of trophic factors or support of RGC axons by other mechanisms has been found to be important. However, modulation of immune activity and promotion of endogenous repair may also play a role. In diseases where the mechanism of action relies mainly on trophic support, stem cells could be either engineered in order to enhance their neuroprotective properties or encapsulated in a removable device to increase safety by localising the graft [114]. Additionally, cells can also be engineered with an inducible suicide gene in order to reduce the risk of any unexpected or otherwise uncontrollable adverse effect [115].

So far, MSCs and OECs are the most successful and widely investigated stem cell type in the field. MSCs have been found to be strongly neuroprotective to RGCs in several pathological conditions of the optic nerve. For instance, MSCs intravitreally transplanted in an in vivo model of experimental glaucoma effectively reduced axonal loss by 60 % [77]. Moreover, in an animal model of optic nerve injury, transplanting MSCs at the site of the optic nerve transaction not only protected RGCs but also induced partial regeneration [116]. Similarly, in an in vivo model of ischemia, intravitreal transplantation of MSCs resulted in an increase in RGC survival by 25 % [117]. The observation that this neuroprotective effect may occur without any physical contact between the graft and the retina suggests that secretion of diffusible neurotrophins may be the main responsible mechanism. Indeed, it has been demonstrated that MSCs transplanted intravitreally or at ON lesion site produce several neurotrophic factors, including CNTF, BDNF, glia cell-derived neurotrophic factor (GDNF) and bFGF [116, 118]. This is of particular relevance for glaucoma, where accumulation of motor proteins is suggestive of disruption of the retrograde transport [31, 47]. Supportive of this hypothesis is the observation that intraocular injection of BDNF in experimental model of glaucoma resulted in decreased RGC loss [48]. As previously mentioned, MSCs can also be manipulated in order to enhance their ability to secrete trophic factors. This effect can be achieved by culturing MSCs with a cocktail of defined factors prior transplantation [119] or by virally transducing MSCs to overexpress neurotrophins. Interestingly, a recent study showed that in a rat model of glaucoma transplantation of MSCs oversecreting BDNF resulted not only in an increased RGC survival but also in functional improvements [120].

Similarly to MSCs, OECs have been shown to protect RGCs by secreting a variety of trophic factors [100, 121]. Moreover, as previously mentioned, other studies suggest that the protective effect of OECs is also mediated by other possible mechanisms, such as cell contact with the host [102, 122]. For all these reasons, OECs and MSCs are generally regarded as two good candidates for neuroprotection. In addition to their properties, they have advantage of an autologous stem cell source, easy to isolate and with no ethical issue or risk of rejection.

6.4.2.2 Cell Replacement and Regeneration

Although increasing RGC survival is a first critical step for a successful cell therapy, in cases of traumatic injury the need of regenerative approaches and cell replacement is indisputable. In addition, glaucoma is another condition where regeneration and cell replacement would be desirable, ideally to restore lost vision. Cell replacement could conceivably be achieved by mechanisms of endogenous or exogenous repair. Endogenous repair could involve modulating the retinal stem cell population or by persuading mature retinal cells to dedifferentiate and redifferentiate into the desired cell type, through a mechanism known as transdifferentiation. For this purpose, progenitor cells of the CMZ and Muller glia have been proposed as potential cell sources. According to recent work, modulation of the membrane plasma potential by application of external stimuli can lead Muller glia [123, 124] and retinal progenitor cell of the CMZ [125] to undergo dedifferentiation and proliferation. Interestingly, the newborn population seems to display neurogenic potential, with markers of mature glia such as glutamine synthetase and vimentin replaced by markers of a progenitor lineage, such as Pax6, Sox3, Nestin and Chx10 [113, 114]. This evidence shed light on the possibility to use neurotransmitters to stimulate retinal endogenous cell replacement and repair.

Alternatively, exogenous repair could be achieved by autologous or heterologous stem cell transplantation into the vitreous or at the injury site of the ON. For regenerative purpose ES cells are currently the most widely used cell source thanks to their relative safety. Indeed, current stem cell trials in macular degenerative diseases all involve transplantation of cells derived from ES cells. However, attention has recently focused on other feasible sources, such as MIO-M1, IPSCs and NPCs. In particular, MIO-M1 and IPSCs may be good candidates for autologous cell transplantation, with less ethical issues and a presumed lower risk of rejection. Alternatively, a potential source of heterologous cells are the NPCs. For neuronal progenitors, high expectations are based on their ability to differentiate into mature neuronal cell type. However, even though all these possible candidates do start expressing neuronal retinal markers once intravitreally transplanted, so far a robust differentiation of the transplant to form functional neurons has not been observed. As an example, transplanted MIO-M1 in a glaucomatous retina only occasionally express the neuronal marker BIII tubulin and, despite the elongated and migratory phenotype displayed, they fail to integrate the retina [107]. Similarly, NPCs, once engrafted intravitreally or at the ON injury site, fail to differentiate into retinal neurons [126], and effective integration into the host tissue remains a considerable challenge.

6.4.3 Challenges to Overcome

Despite the progress made in the identification of potential strategies to prevent or reverse neuronal loss, there are still many barriers to overcome for a successful stem cell therapy (Fig. 6.2).

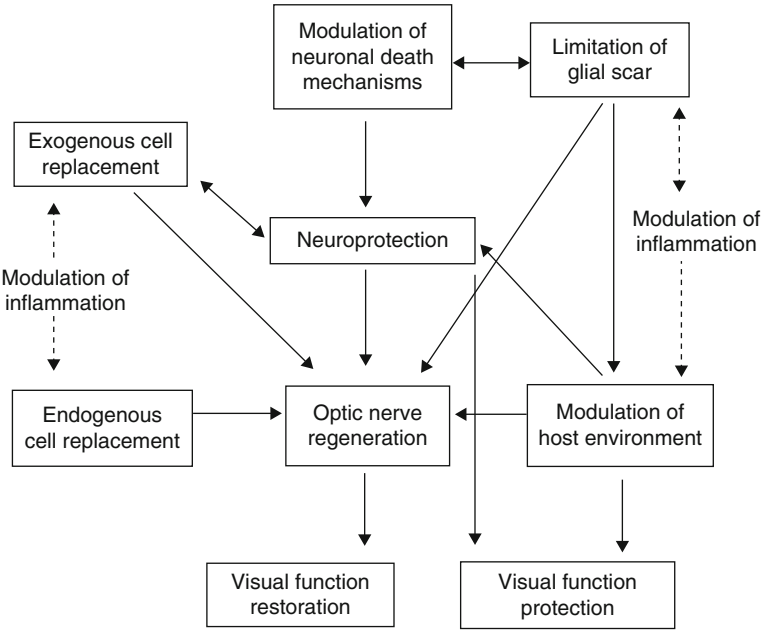

Fig. 6.2 Toward optic nerve regeneration and visual function protection/restoration. Figure adapted from our published work [138]

With regard to neuroprotective strategies, their potential seems to depend mainly on the ability of transplanted stem cells to release trophic factors. However, the secretome of these cells does not consist only in beneficial agents, but might include also deleterious factors. For instance, vascular endothelial growth factor (VEGF) might cause retinal neovascularization [127]. In the same way CNTF, well known to increase neuronal survival and regeneration in degenerative conditions [128], might induce retinal gliosis [129] and alteration of visual function [130].

The responsiveness of the tissue to the graft represents another obstacle. Although one of the advantages of a stem cell therapy is that it provides a long-lasting effect, it is still unknown whether the tissue actually remains responsive to graft-derived trophic factors over the time. For example, exposure of retinal tissue to BDNF results in the downregulation of its receptor TrkB, required to activate intracellular pathways [131]. The identification and removal of any of potential harmful cues and the definition of the therapeutic window during which the tissue is responsive to treatments still represents a limit to overcome in order to potentiate stem cell-mediated neuroprotection.

In terms of RGC regeneration, major barriers are the inhibitory environment characterizing the optic nerve after injury and the intrinsic inability of mature RGCs to grow axons.

Myelin debris with its inhibitory cues, such as Nogo and MAG, and the fibrotic glial scar, characterized by glial fibrillary acid protein (GFAP) overexpression and

CSPG deposition, are the main players in the formation of a hostile extracellular environment limiting any regenerative attempt. Moreover, as previously mentioned, mature neurons have lost their intrinsic ability to regenerate during development. Suppression of gliotic process and restoring RGC ability to regenerate are major challenges for regenerative therapies. Progress has been made in the identification of possible pathways involved in the establishment of these barriers. Potential targets are RhoA/ROCK and JAK/STAT3 signalling pathways, whose inhibition and activation, respectively, results in the creation of a more permissive environment and in a moderate regeneration of the optic nerve beyond the lesion site [63]. However, further investigation is still needed in order to better define eventual detrimental side effects associated with such potential therapeutic approaches. In the meantime alternative strategies have been suggested. For instance, implantation of peptide nanofibre scaffold (SAPNS) [132] or PNF grafts [133, 134], such as sural or Schwann graft, would allow the creation of a permissive bridge through which RGC axon can regenerate unaffected by the surrounding hostile environment.

RGC replacement therapy faces several major limits, not least stem cell differentiation and integration, axonal growth and re-establishment of the retinotopic map. So far several sources of stem cells have been seen to be able to express neuronal markers once transplanted intravitreally; however, none of them seems to complete their differentiation into mature functional RGCs. Muller cells for instance have the potential to differentiate into amacrine, bipolar and photoreceptors, but still there is little evidence of their ability to generate RGCs [103, 105]. In the same way, NPCs, even if already committed to a neuronal fate, also fail to differentiate into neurons once intravitreally transplanted [126]. Whether this is due to the suppression of the RGC differentiation signalling cascade in the mature retina or to the absence of required receptors in undifferentiated immature cell type is not yet known. Guiding the differentiation of these potential stem cell sources in vitro prior to transplantation might be a possible solution, as demonstrated with IPS cells [83]. However differentiation is not the only obstacle. Once suitable RGC precursors have been generated, they need to integrate into the host tissue. In this regard, reactive gliosis represents the major barrier to stem cell migration and engraftment. Indeed, previous data show that suppression of Muller cell reactive gliosis by administration of alpha-aminoadipic acid (AAA) facilitates stem cell integration into the inner retina [135]. However, AAA is toxic and therefore is unlikely to be therapeutically useful. Macrophage and microglia activation also seem to play a role in limiting the migration of transplanted stem cells into the host retina and immune suppression by administration of prednisolone and indomethacin seems to facilitate the engraftment [136]. However, despite the identification of reactive gliosis as major impediment, the degree of integration observed in the inner retina is still modest and not sufficient for a successful replacement therapy. A better understanding of the pathways and molecules involved in the formation of the gliotic barrier is needed.

RGC axon elongation, synaptic connection and re-establishment of the retinotopic map represent the final and perhaps most difficult challenge to overcome for both a regenerative and replacement purpose. Administration of chemoattractants able to guide RGC axons to destination is under investigation. However, so far, there

is no evidence of functional RGC replacement. Nevertheless, research in the field carries on and, although it is not clear yet how difficult will be to overcome this challenge, it is important to keep in mind that even rudimentary reconnection resulting from some level of plasticity achieved by stem cell-based or pharmacological strategies could be beneficial to patients with advanced visual loss.

6.5 Conclusions

To date, the failure of the optic nerve to regenerate remains a major scientific and clinical problem.

A feasible therapeutic strategy could be using a combinatorial approach, consisting for example of gene therapy and stem cell transplantation for cell survival, regeneration and replacement combined with pharmacological treatment for axonal protection and growth. Assuming that one day the intrinsic ability of RGC to regenerate can be restored and that lost RGCs can be successfully replaced, there will still be the need to create a favourable environment for axonal regrowth. In this regard PNS grafts, such as Schwann cell graft or sural nerve grafts, have potentially useful properties as a bridge through which axons can grow, unaffected by the toxicity and the inhibitory features characterizing the environment surrounding the injury site. Administration of chemoattractants could help to re-establish the retinotopic map by driving growing axons from stem cells to the right brain targets. Synaptic integration and function improvement represent the final and most challenging step.

While research carries on in order to achieve such goals, the evidence of structural and functional benefits offered by stem cells even in absence of differentiation, integration and successful replacement in the host tissue is very encouraging. Although neuroprotection will not rescue dead RGCs, it may slow down the progressive deterioration that most of the patients experience over the time. Indeed, even when most of the neurons are already lost, as in endstage glaucoma, a neuroprotective approach may still be beneficial to the surviving host. In addition, the possibility that neuroprotection could increase the receptive field by stimulating dendritic sprouting or by forming functional synapses makes this strategy a promising tool to achieve some degree of visual improvement. So far, neuroprotection appears a more realistic approach for RGC survival and vision preservation in a short term and we predict it is more likely to be translated into clinical treatments more rapidly than any other therapy for RGC regeneration or replacement.

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Chapter 7
Stem Cell Strategies for Diseases of the Outer Retina

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Contents

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A. Pébay (ed.), *Regenerative Biology of the Eye*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4939-0787-8_7,

Abbreviations

ABCA4	ATP-binding cassette, subfamily A, member 4
AMD	Age-related macular degeneration
BEST1	Bestrophin
CHM	Choroideremia
CRD	Cone-rod dystrophy
CRX	Cone-rod homeobox
EFEMP1	Epidermal growth factor-containing fibulin-like extracellular matrix protein 1
ERG	Electroretinography
hESC	Human embryonic stem cells
iPSC	Induced pluripotent stem cells
LCA	Leber congenital amaurosis
MAK	Male germ-associated kinase
OAT	Ornithine aminotransferase
PR	Photoreceptor
PRPH2	Peripherin 2
PSC	Pluripotent stem cell
RCS	Royal College of Surgeon
REP1	Rab escort protein-1
RHO	Rhodopsin
RP	Retinitis pigmentosa
RP1	Retinitis pigmentosa 1
RP9	Retinitis pigmentosa 9
RPE	Retinal pigmented epithelium
RPE65	Retinal pigment epithelium-specific protein 65 kDa
RPGR	Retinitis pigmentosa GTPase regulator
TIMP3	Tissue inhibitor of metalloproteinases-3
USH2A	Usher syndrome 2A
VEGF	Vascular endothelial growth factor

7.1 Introduction

The retina is the light-sensing tissue that lines the inner surface at the posterior part of the eye. Light is perceived by chemical and electrical signals initiated in the retina that stimulate retinal ganglion cells to transmit signals to the visual centres of the brain via the optic nerve. Within the retina, phototransduction is initiated in photoreceptors (PRs), specialised neurons that convert light into electrical signals that are transmitted and ultimately processed by the visual centres within the brain. The health and function of PRs are critically dependent on neighbouring retinal pigmented epithelium (RPE) cells, which separate PRs from the blood supply in the choroid. RPE cells are attached to Bruch's membrane, which acts as a semi-permeable barrier between the

RPE and vasculature of the choroid. The choroid provides the blood supply to the outer retina. RPE cells perform a number of important functions that are essential to the overall homeostasis of the retina which include retinol cycling, nutrient transport, growth factor production, and phagocytosis of PR outer segments [1].

Dysfunction of PRs or RPE can lead to vision loss and often causes irreversible degeneration of other retinal supporting or downstream cells. Retinal degenerative diseases affect millions of people worldwide and have an immense impact on quality of life. Unfortunately the majority of these conditions are currently untreatable. However, through the use of pluripotent stem cells (PSCs), new strategies for studying these diseases offer profound hope of ultimately identifying novel treatments.

Stem cells are unique in that they are capable of both self-renewal and subsequent differentiation into any number of specialised cell types. Stem cells are frequently defined according to their origin and the range or extent to which they can differentiate. PSCs can differentiate into any somatic cell type of the body, whereas multipotent stem cells are somewhat more restricted in the types of cells they can become. PSCs can be derived from various sources and include human embryonic stem cells (hESCs) [2, 3] and induced pluripotent stem cells (iPSCs) [4–6]. A detailed discussion of hESCs and iPSCs can be found in Chap. 5. Together, hESCs and iPSCs (collectively PSCs) provide a novel set of tools for the study and treatment of many diseases through their application in developing cellular models and therapies. Indeed, retinal diseases are currently targeted for clinical trials using PSC-based therapies, demonstrating the exciting possibility that these strategies may in fact translate into clinical outcomes in the near future.

To understand stem cell-based approaches for treating retinal diseases, we begin with a brief summary of the pertinent clinical features of diseases that affect the outer retina (i.e. from the outer plexiform layer to the RPE). A particular focus has been made on diseases where PSCs have been used for either disease modelling or cell therapy and on diseases that are strong candidates for these stem cell strategies given the current state of the field. We then outline the potential of PSC-related therapies for outer retinal diseases.

7.2 Outer Retina Diseases

7.2.1 Age-Related Macular Degeneration

Age-related macular degeneration (AMD) (OMIM #603075, reviewed in [7]) is a multifactorial disorder with both genetic and environmental risk factors and involves progressive degeneration of PRs and underlying RPE cells in the macula, the part of the eye responsible for central vision. The clinical hallmarks of AMD include the accumulation of extracellular deposits, termed drusen, beneath the RPE on Bruch's membrane and pigment abnormalities from dysfunctional RPE cells. Advanced

stages are characterised by central visual loss due to geographic atrophy of the RPE ('dry' AMD) and/or choroidal neovascularisation ('wet' AMD). AMD is the leading cause of blindness in the Western world and the most common cause of acquired visual impairment in the elderly, affecting over seven million people in the US and approximately 1 in 7 people over the age of 50 in Australia [8, 9]. The vast majority of patients have the atrophic, or 'dry', form of the disease, for which there is currently no treatment. A subset of people with atrophic AMD go on to develop exudative, or 'wet', AMD, which is currently managed if diagnosed early by serial injections of anti-angiogenic drugs that block vascular endothelial growth factor (VEGF)-induced neovascularisation [10]. This treatment often halts or slows vision loss and many patients experience restoration in vision with timely intervention.

7.2.2 Stargardt Disease

Stargardt disease (OMIM #248200, reviewed in [11]) is an autosomal recessive, juvenile-onset macular dystrophy caused by mutations in the *ATP-binding cassette, subfamily A, member 4* (*ABCA4*) gene. Clinically it is characterised by loss of visual acuity, though peripheral visual fields remain normal, and rapid progressive degeneration of the macula region of the retina. Histologically, it is characterised by subretinal deposition of lipofuscin-like material in RPE cells and PR segments. Later stages of the disease involve abnormal slowing of the rod and cone retinoid cycle and death of RPE and PRs. There are no treatments available for Stargardt disease.

7.2.3 Best Disease

Best Disease (OMIM #153700, reviewed in [12]) is an autosomal dominant, early onset macular dystrophy frequently caused by mutations in the Bestrophin (*BEST1*) gene [13]. Clinically it is characterised by the bilateral presence of bright yellow lesion containing lipofuscin-like material in the subretinal space that resemble a sunny-side-up egg, termed 'vitelliform', upon examination. In many individuals these lesions eventually rupture, giving a 'scrambled egg' appearance and leading to deposits and fluid in the affected area of the macula, pigment abnormalities, atrophy of the underlying RPE, and progressive reduction in central vision. Unfortunately there are currently no treatments for this retinal dystrophy.

7.2.4 Doyne Honeycomb Retinal Dystrophy

Doyne Honeycomb Retinal Dystrophy (OMIM #126600, reviewed in [14]) is an inherited disorder predominantly caused by mutations in the epidermal growth factor-containing fibulin-like extracellular matrix protein 1 (*EFEMP1*) gene.

Clinically, it resembles AMD, with sub-RPE drusen developing in early adult life and a progressive irreversible loss of central vision. Build up of large drusen, which generally forms a honeycomb-like pattern within the macula, causes progression of the disease. Unfortunately, there are no means by which to definitively treat this uncommon retinal dystrophy.

7.2.5 Retinitis Pigmentosa

Retinitis pigmentosa (RP) (OMIM #268000, reviewed in [15]) is a heterogeneous group of ocular diseases which are clinically characterised by progressive loss of central or peripheral vision and night blindness, secondary to degeneration of the RPE and PRs. Most cases of RP are monogenic. To date more than 50 genes have been identified to cause RP, including rhodopsin (*RHO*), Usher syndrome 2A (*USH2A*), and retinitis pigmentosa GTPase regulator (*RPGR*), which collectively account for approximately 30 % of all cases [16, 17]. To date there is no means by which to definitively treat this blinding condition.

7.2.6 Sorsby Dystrophy

Sorsby Dystrophy (OMIM #136900, reviewed in [18]) is a fully penetrant, autosomal dominant disorder caused by missense mutations in the tissue inhibitor of metalloproteinases-3 (*TIMP3*) gene. Clinically it is characterised by bilateral loss of central vision due to subretinal neovascularisation and RPE atrophy at the macula. Similar to other retinal dystrophies currently there are no means by which to definitively treat this disease.

7.2.7 Cone-Rod dystrophy

Cone-rod dystrophy (CRD) (OMIM #120970, reviewed in [19]) is a progressive retinal degenerative disease which can be inherited in an autosomal dominant, recessive or X-linked pattern. It can be caused by mutations in a number of different genes, including cone-rod homeobox (*CRX*), *ABCA4*, and others. Clinically, it manifests by progressive vision impairment typically beginning with loss of colour vision, reduced visual acuity and sensitivity to light, followed by night blindness and loss of peripheral visual fields. Histologically CRD is characterised by degeneration, and eventually a complete loss, of outer nuclear layer PRs (generally either cones proceeding rods or vice versa). Upon examination, pigment abnormalities and atrophy of the RPE may also be observed in addition to abnormal cone function on electroretinography (ERG), a test that measures the electrical response of cells in the retina. Currently there is no treatment for CRD; however, tinted lenses and low vision aids may help with managing symptoms.

7.2.8 Leber Congenital Amaurosis

Leber congenital amaurosis (LCA) (OMIM #204000, reviewed in [20]) comprises a group of autosomal recessive early onset childhood retinal dystrophies caused by mutations in a number of different genes. Clinically, it is characterised by vision loss, nystagmus, and severe retinal dysfunction often manifesting in the early postnatal period. Progressive degeneration in the cellular structure of the retina causes ERG responses to be severely attenuated or non-recordable and may also lead to structural changes in the cornea that cause it to thin and adopt a conical shape, further distorting vision [20]. Most forms of LCA involve severe degeneration and death of PRs and have no available treatments. A rare form of LCA caused by mutations in retinal pigment epithelium-specific protein 65 kDa (*RPE65*) (OMIM #204100) results in dysfunctional, but relatively preserved, retinal cells. Mutations in this gene cause a deficiency in retinoid isomerase, which leads to a biochemical blockage of the retinoid cycle and degeneration of PRs. Gene therapy trials aimed at restoring the visual cycle in surviving PRs via adeno-associated virus delivery of *RPE65* have shown partial reversal of the dysfunction, although the reconstituted retinoid cycle is not completely normal and PR degeneration still occurs [21, 22]. Importantly though, patients who received gene therapy have shown remarkable and lasting improvements in visual function despite ongoing loss of PRs [21, 22].

7.2.9 Gyrate Atrophy

Gyrate atrophy (OMIM #258870, reviewed in [23]) is an autosomal recessive disorder characterised by slowly progressive atrophy of the choroid, RPE, and retina. Mutations in the ornithine aminotransferase (*OAT*) gene are known to cause gyrate atrophy, and dietary restriction arginine has been shown to halt visual loss [24].

7.2.10 Choroideremia

Choroideremia (OMIM #303100, reviewed in [25]) is an X-linked disease caused by mutations in the choroideremia (*CHM*) gene, which encodes Rab escort protein-1 (REP1), that lead to degeneration of the choriocapillaris, RPE, and PRs. All known *CHM* mutations produce truncated protein products, resulting in a complete loss of functional REP1 protein. In affected males, it is characterised by nyctalopia, progressive loss of peripheral and central vision as a result of complete atrophy of the choroid and retina. Heterozygous females have no visual defect, but may exhibit pigment abnormalities and atrophy around the optic disc. Unfortunately there is no effective treatment for CHM.

7.3 Induced Pluripotent Stem Cells for Retinal Disease Modelling

The extreme difficulty in obtaining ocular tissue from living people currently represents a major barrier to studying the molecular mechanisms of blinding disease. The ability to generate iPSCs from patients with specific diseases provides an extremely powerful means to investigate the underlying pathogenesis. Generating iPSCs directly from patients with a particular disease allows cells to be differentiated into specific cell types for disease modelling, drug screening, and understanding fundamental mechanisms underlying cell biology.

Despite the relatively large of number diseases affecting the outer retina, to date there have only been a small number of studies describing the development and characterisation of patient-specific iPSCs (Table 7.1). This is compounded further by the relatively large degree of genetic heterogeneity amongst these diseases. Despite this, particular insight in the pathogenesis of retinitis pigmentosa 9 (*RP9*)-related RP has been made, whereby *RP9* mutations appear to cause disease, at least in part, through oxidative stress pathways [26]. Conversely, *RHO* and *USH2A* mutations are associated with an increase in endoplasmic reticulum stress [26, 27].

7.4 Pluripotent Stem Cells for Retinal Cell Replacement

Although a number of genetic mutations and variants have been identified that cause or confer risk for diseases of the outer retina, in many cases the disease mechanisms remain poorly understood. Few treatment options exist to preserve or restore vision for a majority of these diseases, and available treatments may only treat symptoms rather than the underlying disease cause. However, the cell types whose degeneration and/or dysfunction lead to vision loss in most cases are known: predominantly RPE, PRs, or a combination thereof. One potential option for treatment involves replacing the degenerative or dysfunctional cells within the outer retina with new healthy cells to restore function and, hopefully, improve vision. Transplanted cells may also protect endogenous retinal cells from further degeneration, minimising future vision loss. This approach, termed cell replacement therapy, is an attractive strategy for many retinal diseases because the population of cells that are defective or have degenerated are generally well characterised and, surgically, the eye is easily accessible. Moreover, as an immune-privileged site, the eye should have a low risk of rejecting transplanted material [28], though results from early clinical trials with allogenic foetal RPE transplants indicate that immunosuppression may still be required if the blood–retinal barrier is compromised due to disease [29–31].

For cell replacement therapy to be feasible, one needs a readily available cellular source from which to generate sufficient numbers of healthy retinal cells for transplantation. Transplant of foetal tissue has shown some promise in a clinical setting [32, 33], but this material is difficult to obtain. As described previously, PSCs can

Table 7.1 Currently described induced pluripotent stem cell lines generated for modelling diseases of the outer retina

References	Disease	Gene	Number of patients included	Disease causing variants studied	Reprogramming factors used	Differentiated cells of interest	Phenotype observed
[82]	Best Disease	*BEST1*	2	(1) A146K (2) N296H	KOSM	RPE	*BEST1* mutant RPE cells show disrupted fluid flux and increased accrual of PR outer segments compare to cells derived from unaffected siblings
[83]	Choroideremia	*CHM*	2	(1) R555stop (2) L550P	KOSM + miRNA 302/367	iPSCs	Reduced REP1-mediated enzymatic activity
[84]	Gyrate Atrophy	*OAT*	1	A226V[a]	KOSM + NANOG, LIN28, SV40 large T-antigen	iPSCs	Assessment of mutational load acquired during gene correction
[45]	Gyrate Atrophy	*OAT*	1	A226V[a]	KOSM + NANOG + LIN28	RPE	Reduced OAT enzymatic activity
[85]	LCA	–	2	–	KOSM	RPE; NSC	Expression differences identified
[35]	LCA	–	1	–	KOSM	RPE	–
[35]	RP	–	2	–	KOSM	RPE	–
[86]	RP	*MAK*	1	Alu repeat insertion in exon 9	KOSM	Retinal progenitor cells	Novel exon transcripts were identified from differentiated retinal progenitor cells
[87]	RP	*RHO*	1	G188R	KOSM	RPE, retinal progenitor cells	RHO is diffusely distributed with expression of endoplasmic reticulum stress markers
[27]	RP	*USH2A*	1	R4192H and pseudoexon IVS40	KOSM	Retinal progenitor cells	Mutations appear to cause disease through protein misfolding and endoplasmic reticulum stress
[26]	RP	*RP1*	1	721Lfs722X	KOSM	Photoreceptors	–
[26]	RP	*PRPH2*	1	W316G	KOSM	Photoreceptors	–
[26]	RP	*RHO*	1	G188R	KOSM	Photoreceptors	RHO mutation is associated with endoplasmic reticulum stress
[26]	RP	*RP9*	2	H137L	KOSM	Photoreceptors	RP9-retinitis pigmentosa is involved, at least in part, in oxidative stress pathways

KOSM KLF4, OCT4, SOX2, and c-MYC, *RPE* retinal pigmented epithelial cells, *PR* photoreceptors, *miRNA* micro-RNA, *NSC* neural stem cell
[a]Same patient sample

be expanded indefinitely in vitro and can also potentially be differentiated into any cell type in the body, including retinal cells; thus, they provide an unlimited and renewable source of cells for transplant. Furthermore, methods to differentiate PSCs to functional RPE [34–39] and PRs [40–46] are well established.

7.4.1 Moving Towards Stem Cell-Based RPE Cell Therapy

The aim of PSC-based cellular therapy is to ultimately replace degenerative retinal cells with new healthy cells that survive, integrate, and remain functionally active long term. As proof of principle, it has been shown that RPE cells can survive post-transplantation and improve visual function in rodent models of retinal degeneration [47–50]. Similarly, human PSC-derived RPE can functionally integrate and improve visual function in rodent models of retinal degenerative diseases [51, 52]. In a mouse model of RP (*Rpe65*$^{rdl2/rdl2}$), human iPSC-derived RPE cells survived long term and improved retinal function over the lifetime of the mice [52]. In the dystrophic Royal College of Surgeon (RCS) rat in which a primary defect in RPE phagocytosis leads to PR degeneration, one study found that iPSC-derived RPE did not survive beyond 13 weeks; however, long-term visual function was maintained, suggesting the effect may be due to a secondary host response [51]. In another study, hESC-derived RPE survived long term (>100 days) following subretinal injection into RCS rats and led to reduced PR degeneration and preserved visual function [53]. Whether visual improvement observed with transplanted PSC-derived RPE is due to bona fide functional cell replacement or indirect paracrine effects remains to be determined. Nonetheless, PSC-based RPE cell therapy appears very feasible.

Towards this goal, phase I/IIa clinical trials of cell replacement therapy for AMD and Stargardt disease are currently underway using allogenic hESC-derived RPE cell transplants [54] (NCT01345006, NCT01344993, NCT 01469832, Advanced Cell Technology; and NCT01674829, CHA Bio and Diostech). IPSC technology has the added advantage of allowing for generation of patient-matched cells for autologous transplant to mitigate the need for immunosuppression. Recently, the first iPSC-derived RPE clinical trials were approved for AMD in Japan (RIKEN). It is important to note that for diseases caused by specific Mendelian mutations, gene correction may be required in iPSCs from the affected patient prior to transplant.

7.4.2 Feasibility of Photoreceptor Cell Therapy

Cell replacement therapy for PRs has not yet advanced into clinical trials; however, promising results from animal studies suggest this may be feasible in the near future. Proof of principle experiments demonstrate that rod precursor cells isolated from postnatal mice can survive transplant, integrate and differentiate into mature PRs, and improve visual function in mouse models of PR dysfunction (*Gnat*$^{-/-}$) [55] and rod degeneration (*Rd1* [56] and *Rho*$^{-/-}$ [57]). HESC-derived retinal progenitor

cells also can survive transplant, differentiate to functional PRs, and improve visual responses in a mouse model of LCA (*Crx*$^{-/-}$ mice) [58]. Similarly, iPSC-derived retinal progenitor cells integrate and differentiate into PRs in vivo [43].

One complicating factor for potential PR replacement therapy in humans is that in many retinal diseases involving PR degeneration, the RPE is often implicated as well. Thus, it is likely that PR cell transplantation may need to be conducted in combination with RPE cells in a dual replacement strategy. Towards this goal, efforts to construct a two-layered patch graft of RPE and PRs are underway that utilise a thin plastic film to anchor a monolayer of PSC-derived RPE cells [59] with a second layer of PR precursor cells adhered via a biodegradable gel [60]. This research is still in the early stages of development. Other efforts to generate striated tissue constructs containing RPE and PRs from PSCs in vitro have been reported via self-assembled optic cup [61] and optic vesicle-like structures [45, 62] and retinal progenitor sheets [63].

7.4.3 Bioengineered Substrates for Cell Transplants

Native RPE exist as a polarised monolayer, and this cellular architecture is critical to their function. Previous studies in animals have demonstrated that sheets of retinal cells survive better following transplantation than dissociated cells [64]. Furthermore, RPE may fail to survive or function on damaged Bruch's membrane, which is a common feature of ageing and some retinal diseases such as AMD [65, 66]. Given these concerns, artificial substrates on which to seed RPE cells are being developed to facilitate transplant of intact, polarised sheets of cells. These include polyester membranes [67], ultrathin parylene films [59], plasma polymers [68], and polyimide membranes [69]. Current clinical trials deliver hESC-derived RPE cells as suspensions via subretinal injection, but a clinical trial application has been submitted to transplant hESC-derived RPE immobilised on a polyester membrane to address this potential issue (NCT01691261) [70].

7.4.4 Pluripotent Stem Cells Recapitulate Retinal Ontogeny

One further advantage of using PSC-derived retinal cells for transplantation is the ability to generate cells at various ontogenetic stages of development. This is important because studies have shown that human foetal RPE and early postnatal mouse PRs function significantly better in vivo than the same respective cells isolated from older tissue [56, 57, 71]. HESC- and iPSC-derived PRs behave similar to early postnatal mouse PRs when transplanted into mice [43, 58]. Likewise, hESC-derived RPE resemble human foetal RPE in vitro and in vivo [36, 72–75], albeit with some differences in growth factor expression and attachment to Bruch's membrane [66]. In both cases however, published results demonstrate the feasibility of generating PSC-derived retinal cells that functionally resemble early developmental stages most useful for transplantation.

7.4.5 *Stem Cell Transplants for Trophic Support*

It is conceivable that transplanted cells could produce trophic factors that provide a neuroprotective effect in the retina without functional integration [76]. This strategy of transplanting cells to provide paracrine support has shown improved visual outcomes in animal models of retinal degeneration using mesenchymal stem cells [77] and umbilical tissue-derived stem cells [78]. Both of these types of stem cells are not pluripotent, meaning they are restricted in the range of cell types they can generate and may not be capable of becoming retinal cells. Mesenchymal stem cells can be obtained from various adult tissues and have an innate ability to home to a site of injury and mitigate endogenous tissue repair in part through modulation of the immune response (reviewed in [79]). However, their ability to differentiate into functional, mature retinal cells remains questionable [80, 81]. Thus, these non-PCSs may be ineffective for replacement therapy. However, transplanting cells to provide trophic support to the retina is a practical treatment strategy, and there are currently several clinical trials underway using cells isolated from bone marrow (NCT01531348) and umbilical tissue (NCT01226628) for RP and atrophic AMD, respectively.

7.4.6 *Receptivity of the Diseased Retina*

A final requirement for cell replacement therapy is that the diseased environment must allow for integration and function of transplanted cells in the retina. One particular concern for retinal diseases with complex or unknown genetic influences (such as some types of RP) or with strong environmental influences (such as AMD) is that degeneration may be an indirect effect of complex or yet unknown disease processes rather than an intrinsic defect in the retinal cells themselves. If this is the case, then it is conceivable that transplanted retinal cells may also succumb to the diseased environment and eventually die along with endogenous cells if the underlying cause of the disease is not addressed. Nevertheless, if cell therapy significantly delays this degenerative process then it will serve as a valuable treatment option.

7.5 Concluding Remarks

In summary, the development of stem cell strategies to treat retinal diseases offers exciting possibilities for the future. PSC-derived RPE cells have now progressed into clinical trials, while the ability to create in vitro human models using iPSCs has revolutionised the field by providing a platform to study disease pathogenesis and to screen therapeutic compounds. There are still many unanswered questions, including whether multigenic diseases or those with unknown genetic, strong environmental or epigenetic influences can be modelled effectively with iPSCs. It also remains to be

determined whether improvements from cell therapies in animal models will translate to human conditions and whether the diseased retina will facilitate long-term function of cell transplants. Regardless of these uncertainties, stem cell approaches provide hope for new insight and treatments for a large number of retinal diseases.

Acknowledgments This work was supported by funding from the National Stem Cell Foundation of Australia, the Ophthalmic Research Institute of Australia, and the Australian National Health and Medical Research Council (Project Grant 1059369 and a Centres of Research Excellence Grant 1023911). AWH is supported by an Australian National Health and Medical Research Council Peter Doherty Fellowship. CERA receives Operational Infrastructure Support from the Victorian Government.

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Chapter 8
Potential of Müller Glia and Stem/Progenitor Cells to Regenerate Retinal Tissue

Marius Ader, Volker Enzmann, and Mike Francke

Contents

Abbreviations

ASC	Adult stem cell
AMD	Age-related macula degeneration
MC	Müller cell
NSC	Neural stem cell
PDGF	Platelet-derived growth factor

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A. Pébay (ed.), *Regenerative Biology of the Eye*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4939-0787-8_8,

PN Postnatal day
RP Retinitis Pigmentosa
RPE Retinal pigment epithelium
RSC Retinal stem cell
SC Stem cell

8.1 Introduction

Regenerative medicine includes several therapeutic strategies to replace degenerated tissue and/or cells or to restore physiological functions: promotion of endogenous regeneration via therapeutic use of growth factors, exogenous delivery of allogeneic or autologous living cells, and tissue engineering with the development of artificial tissues and/or entire organs. The use and application of stem or progenitor cells to replace damaged tissue represent highly promising approaches with currently remarkable results, at least in animal models [1]. Several types of stem and progenitor cells for cellular therapies have to be discriminated [1, 2]. During normal ontogenesis of all vertebrates the body develops from a totipotent stem cell (zygote) via pluripotent stem cells (e.g., in blastocyst stadium) to organ constituting progenitor cells. In the adult body several types of adult stem cells (ASCs) remain in some organs. All these different stem and/or progenitor cells have different potentials for self-renewing, proliferation, survival, and cell type differentiation.

The relative easy accessibility of the eye and the possibility of direct visual control of the surgery due to the translucent cornea and lens make the retina one of the prime regions for evaluating neuronal replacement strategies in the CNS. The retina is a well organized neural structure with defined retinal layers, cell types, and cellular components (Fig. 8.1). Furthermore, the relative simple structure and the well known function of photoreceptors have many advantages compared to complex neurons in brain tissue. Photoreceptors as uni-directional sensory neurons only have to establish a short “axon”, minimizing problems associated with long axonal growth, as in the case of motor neurons in the brain. Furthermore, they have to develop only a single synaptic contact, and as sensory neurons obtaining input by light do not have to generate large, complex synapse-covered dendritic trees that have to receive multiple proper inputs in case of many interneurons. Indeed, since more than two decades the mammalian retina has been extensively used for cell transplantation experiments with the aim to replace photoreceptors beside other retinal cell populations including retinal pigment epithelium (RPE) cells or retinal ganglion cells (RGCs) [3, 4].

Photoreceptor loss as it is observed in several retinal degenerative diseases including AMD, RP, or cone-rod dystrophies leads to vision impairment and blindness. Some of these diseases are more complex and include also degeneration of the RPE. In mammals, including humans, degenerated retinal cells are permanently lost, as the adult mammalian retina has no intrinsic regenerative capacity. Currently, no effective treatments are available for such conditions. Four main approaches for restoring light detection and vision following complete loss of photoreceptors are

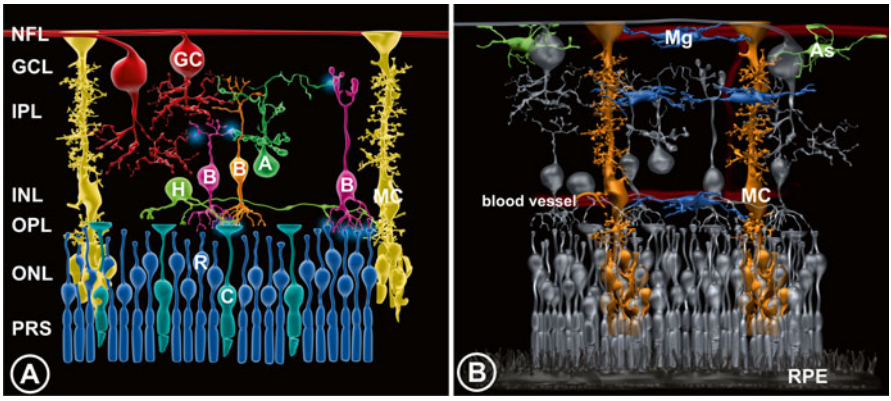

Fig. 8.1 Schematic drawing of the layer structure of a vertebrate retina with different neuronal cell types (**a**) and (**b**) all non-neuronal cell types (i.e. different glial cell types and pigment epithelium cells) of a vascular mammalian retina. *NFL* nerve fiber layer, *GCL* ganglion cell layer, *IPL* inner plexiform layer, *INL* inner nuclear layer, *OPL* outer plexiform layer, *ONL* outer nuclear layer, *PRS* photoreceptor segments, *GC* ganglion cell, *B* different types of bipolar cells, *A* amacrine cell, *H* horizontal cell, *R* rod photoreceptor, *C* cone photoreceptor, *MC* Müller glia cell, *As* astrocyte, *Mg* microglial cell, *RPE* retinal pigment epithelium

currently under investigation: (1) intrinsic regeneration, (2) artificial retinal implants, (3) optogenetic approaches, and (4) cell transplantation. Although regeneration by intrinsic cells like Müller glia and RPE cells, light detecting artificial implants or the use of inner retinal neurons (e.g., bipolar or ganglion cells) transformed into light-sensing cells by expression of channel- or halorhodopsin represent highly innovative strategies, the transplantation of "true" photoreceptors might have advantages in regard to sensitivity, resolution, synaptic connections, and proper signal processing within the retinal circuitry (see below).

Besides the potency of progenitor or Müller glial cells, RPE cells possess two known properties which are crucial for their potential role as source for cell replacement in retinal degeneration: proliferation and plasticity in the adult eye. RPE cells originate from the same neuro-ectodermal germ layer tissue as the neuronal and glial progenitor cells for the development of the retinal tissue. Several dedifferentiation processes of pigment epithelial cells have been described during various retinal diseases and therefore, it might be feasible to use RPE cells as a cellular source for retinal regeneration [1, 2].

8.2 Müller Cells and Their Potential as Progenitor Cells

The vertebrate retina contains several main types of neurons: light-sensitive photoreceptors, different types of bipolar and amacrine cells, horizontal and ganglion cells (Fig. 8.1a). Generally, many vertebrates and the mammalian retina contain three types of glial cells. In addition to microglial cells, there are two forms of

neuron-supporting macroglial cells, astrocytes, and Müller (radial glial) cells [5]. As an exception, oligodendrocytes can be found in the myelinated nerve fiber bundles ("medullary rays") of rabbits and hares and the avian and fish retina possess few myelinating oligodendrocytes, as a fourth type of glia. Microglial cells are the blood-derived resident immune cells within the retina and are involved in inflammatory processes, neurodegeneration, and tissue repair [6, 7]. In species with completely or locally vascularized retinae, astrocytes are also located in these innermost retinal layers (in avascular retinae/retinal areas, they are absent). The Müller glial cell (MC) is the principal glial cell of the vertebrate retina; in the avascular retinae of many vertebrates (including mammals) it constitutes the only type of macroglial cells [8]. Müller cells are specialized radial glial cells which span the entire thickness of the retina (Fig. 8.1b) and contact/ensheath all retinal neuronal somata and processes. Thus, Müller cells constitute an anatomical, physiological, and functional link between the retinal neurons and glia cell compartment and MCs are involved in a lot of metabolic and cell-physiological interactions [8].

The ontogenetic development in the vertebrate retina comprised of two main proliferative phases—an early and a late phase—for generating all neuronal and glial cell types [5]. In the early phase mainly cones, amacrine cells, and ganglion cells are generated (photopic pathway), followed by the late phase with rods, horizontal cells, bipolar cells, sub-populations of amacrine cells (scotopic pathway), and Müller glial cells. However, there are no glia- or neuron-specific progenitor cells; even the final division of a late retinal progenitor cell typically generates one rod photoreceptor or bipolar cell and one Müller cell [5, 9]. This means, there exists a common progenitor cell type for both neurons and Müller glial cells and it might be that dedifferentiated Müller glial cells could re-establish the progenitor cell properties [10, 11]. The gene expression profiles of retinal Müller glia and mitotic progenitor cells were found to be highly similar in the developing mouse retina, suggesting that Müller glia might serve to produce multiple retinal cell types under the right conditions [12].

The mature mammalian retina is thought to lack intrinsic regenerative capacity. However, capacity for retinal regeneration in cold-blooded vertebrates has long been recognized [13]. In fish and amphibians, the retinal stem cells continue to produce progenitors throughout life, adding new retina to the periphery of the existing retina as the eye grows. Additionally, regeneration occurs in fish retinas through a population of retinal stem cells residing at the peripheral margin of the retina [14]. Furthermore, complete MC-dependent regeneration has been observed in the zebrafish after pharmacologically induced photoreceptor degeneration [15]. It has generally been thought that homeothermic vertebrates, such as birds and mammals, lack this so-called ciliary marginal zone. However, there is evidence that the retina of postnatal chickens has the potential to generate new neurons [16]. In response to acute damage, numerous Müller glial cells re-entered the cell cycle, and shortly thereafter, expressed CASH-1, Pax6, and Chx10, transcription factors expressed by embryonic retinal progenitors. Some of these newly formed cells differentiated into retinal neurons, a few formed Müller glia, and most remained undifferentiated, with continued expression of Pax6 and Chx10 [16]. Furthermore,

stem cells in the adult mouse eye were identified, which represents a possible substrate for retinal regeneration [17]. Single pigmented ciliary margin cells clonally proliferate in vitro to form sphere colonies of cells that can differentiate into retinal-specific cell types, including rod photoreceptors, bipolar neurons, and Müller glia. Adult retinal stem cells are localized to the pigmented ciliary margin, indicating that these cells may be homologous to those found in the eye germinal zone of other non-mammalian vertebrates.

In response to virtually every pathological alteration of the retina, including light damage, retinal trauma, ischemia, retinal detachment, glaucoma, diabetic retinopathy, and age-related macular degeneration, Müller cells become reactivated [18]. Reactive gliosis includes morphological, biochemical, and physiological changes of Müller cells; these alterations vary with type and severity of insult [18–20]. Müller cells may dedifferentiate to progenitor-like cells, and a subsequent (restricted) transdifferentiation to cells with neuronal phenotype may participate in tissue regeneration [5, 21]. After retinal injury, a population of Müller cells dedifferentiates to cells with properties similar to multipotent retinal progenitor/stem cells and expresses neuronal and photoreceptor proteins [19, 22, 23]. In retinas with toxicologically damaged retinal neurons, a subset of proliferating Müller cells differentiated into amacrine cells, as defined by the expression of amacrine cell-specific markers [24]. However, the neuron-regenerating potential of Müller cells in situ is very restricted, especially in mammals. Attempts to facilitate the neurogenic program of Müller cells, e.g., by transdifferentiation of cultured Müller cells, are ongoing [19, 25]. Due to their potential for proliferation and for generating neural progenitor/stem cells, Müller cells will have a great impact on future cell-based therapeutic approaches. However, suitable sources to obtain enough potential Müller glial cells, identification of molecular signals that trigger the neurogenic process in vitro or in vivo and to increase the number of newly generated neurons remain main problems.

8.3 Stem Cell-Derived Photoreceptors for Cell-Replacement Strategies

8.3.1 Proof-of-Concept: Transplantation of Primary Photoreceptors

The adult mammalian retina, as most parts of the adult mammalian CNS, represents a non-neurogenic tissue that does not show generation of new neurons or their migration and integration into the tissue during “normal” life or following degeneration/injury. These conditions lead to the fundamental question what type of transplanted donor cells might have the potential to correctly integrate into the adult mammalian retina and generate mature photoreceptors that form synaptic connections to endogenous second-order neurons.

By using primary photoreceptors isolated from different developmental stages of the mouse retina the proof-of-concept of photoreceptor transplantation was evaluated [26, 27]. Starting with pilot work more than 20 years ago first evidences for successful integration and photoreceptor maturation were demonstrated following transplantation of photoreceptors isolated from the developmental mouse retina into mouse models of retinal degeneration [28–30]. Further detailed analysis using donor cells isolated from fluorochrome-expressing reporter mice revealed that cells from postnatal-day (PN) 4/5 retinas yielded the highest integration rate [26, 27]. Interestingly, further analysis of the donor cells using photoreceptor-enriched cell suspensions and BrdU labeling demonstrated that young, post-mitotic photoreceptors rather than multipotent retinal progenitor cells have the capacity for retinal integration [26, 31]. However, mature photoreceptors might also still have the potential for integration but at very low rates, possibly due to strongly increased cell death [32].

Donor photoreceptors integrate into the outer nuclear layer (ONL) of wild-type hosts and develop the characteristic morphology of mature photoreceptors including a nucleus-containing cell body within the ONL, a spherule-like axonal terminal in the outer plexiform layer that expresses pre-synaptic markers and inner- and outer-segment-like structures above the outer limiting membrane within the subretinal space (e.g., Fig. 8.2). Besides the photoreceptor-specific expression of fluorochromes, immunohistochemical analysis using photoreceptor-specific markers demonstrated the generation of mature photoreceptors from transplanted donor cells [26, 27]. Importantly, ultra-structural investigations revealed the formation of native, discs-filled outer segments by donor photoreceptors, an indispensable prerequisite for proper light detection [27, 33]. Indeed, recent data demonstrated light-sensitivity of donor photoreceptors and their functional integration into the

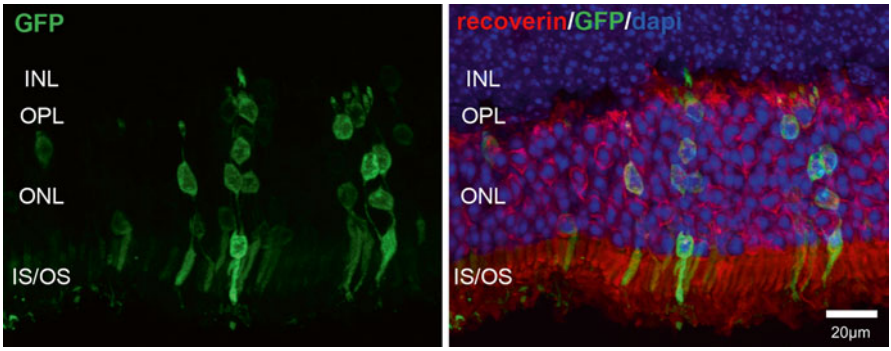

Fig. 8.2 Photoreceptors (*green*) isolated from a GFP reporter mouse at postnatal day 4 correctly integrated into the ONL after transplantation into an adult wild-type mouse. Donor cells showed the characteristic morphology of mature photoreceptors with synaptic terminals in the outer plexiform layer, a nucleus-containing cell body in the ONL, and apically located inner-/outer segments (IS/OS). Donor and host photoreceptors expressed the photoreceptor marker recoverin (*red*). *INL* inner nuclear layer, *ONL* outer nuclear layer

host neural circuitry. At the single cell level, transplanted photoreceptors showed light-driven translocation of proteins between cell body and outer segment [33, 34] besides electrophysiological and Ca^{2+} responses to light stimulation [34, 35]. Furthermore, following transplantation into mouse models of retinal degeneration some behavioral improvements could be detected [34–37]. Indeed, the generation of discs-filled outer segments was also observed after transplantation of photoreceptors into heavily degenerated retinas that no longer allowed proper tissue integration due to the almost complete loss of the ONL [33]. However, besides these promising results and the demonstration for the proof-of-concept of photoreceptor replacement in the adult mammalian retina, there are still several issues to be considered before such cell-based strategies can be translated towards clinical applications: (1) the number of integrating donor cells is still too low for proper vision and has to be significantly increased; (2) although some transplanted photoreceptors survived long-term (up to 1 year) in mouse recipients their number significantly decreased over time; therefore, the mechanisms of donor cell survival and possible immune-responses have to be investigated; (3) synapse formation of all grafted photoreceptors has to be analyzed in detail to evaluate connections to bipolar and horizontal cells and proper functional integration within ON and OFF pathways, (4) human vision mainly depends on cone photoreceptor-mediated day-light (photopic) vision allowing color detection and high acuity; thus the potential of cones for transplantation has to be evaluated.

Currently, the majority of data regarding photoreceptor transplantation has been collected from experiments in which primary cells from the developmental mouse retina have been used. However, the developmental stage for optimal integration of mouse photoreceptors, i.e., PN4/5, corresponds to the second trimester in human development, therefore strongly limiting the access to considerable amounts of donor material for clinical trials. Therefore, an in vitro expandable cell source with the potential to generate high amounts of transplantable photoreceptors will be mandatory for the development of photoreceptor replacement therapies. Indeed, several stem and progenitor cell populations have been analyzed for their proliferation in culture and potential to differentiate along retinal and photoreceptor lineages followed by transplantation studies into the mammalian retina [4].

8.3.2 Retinal Stem/Progenitor Cells

In the retina all neuronal subtypes, including photoreceptors, and Müller glial cells originate from multipotent retinal progenitor cells [38]. Following up on findings for the in vitro growth of multipotent neural stem cells (NSCs) isolated from the developmental or adult brain [39, 40], several studies also evaluated the stem cell properties of in-culture expanded retina-derived cells. These approaches were based on the hypothesis that tissue-specific retinal stem cells can be, due to their retinal origin, more easily directed towards specific retinal phenotypes like photoreceptors

than NSCs generated from other CNS regions. Indeed, by using high concentrations of the mitogens FGF-2 and/or EGF, in vitro expandable retinal stem/progenitor cell (RSC) lines have been established [17, 41–46]. Such RSC lines were generated from cells isolated either from the developmental neuro-sensory retina or the pigmented ciliary margin of adult donors. Interestingly, RSCs were not only established from laboratory animals including mouse, rat, and pig [17, 41, 44–47], but also from human tissue [48, 49]. RSCs can be grown as free-floating neurospheres or as monolayers and exhibit the cardinal characteristics of multipotent stem cells, that is self-renewal and differentiation into multiple cell-types. Thus, RSCs expressed typical markers for NSCs including nestin, Pax6, Sox2, and members of the notch pathway like Notch1, Hes1, and Hes5 during proliferative expansion. Upon growth-factor withdrawal RSCs differentiated into multiple cell-types including GFAP-expressing glial cells and ß-III-tubulin and Map2 expressing neurons [17, 41, 44–47]. Interestingly, some studies also observed the differentiation of RSCs along the retinal lineage including cells that expressed rhodopsin, recoverin, or peripherin, markers characteristic for photoreceptors. Furthermore, by modulation of culture conditions [44] or over-expression of photoreceptor-specific transcription factors [50, 51], some studies described an increase in the number of RSC-derived photoreceptors. RSCs have been used for transplantation studies into murine and porcine retinas [41, 45, 48]. Here, RSC-derived donor cells showed extensive integration within the retinal tissue and some studies suggested the generation of rhodopsin, recoverin, or Rom1 expressing photoreceptors [41, 48] that lead to some functional improvements in mouse models of retinal degeneration [41, 51]. However, none of these studies provided a detailed analysis of the photoreceptor phenotype of transplanted cells that actually did not resemble the morphology of mature photoreceptors as it was shown for transplanted primary photoreceptor cells [26, 27, 33, 34]. Indeed, several studies recently suggested that in vitro cultured RSCs do not resemble a retinal progenitor phenotype [45, 52, 53]. In these contradicting reports RSCs derived from the pigmented ciliary margin of adult mice failed to differentiate along the photoreceptor lineage. As the authors showed, such cells retained their pigmented epithelial identity without developing a RSC phenotype despite expression of nestin during proliferative expansion and up-regulation of pan-neuronal markers like ß-III-tubulin following differentiation [52, 53]. Furthermore, also in vitro generated RSCs isolated from the developing mouse neuroretina lost the expression of characteristic retinal progenitor cell transcription factors including Rx or Chx10 [45] and failed to differentiate along the photoreceptor lineage [45, 54]. Surprisingly, such RSCs were able to differentiate into myelinating oligodendrocytes [45], a cell-type not generated by retinal progenitor cells in vitro or in vivo [45, 55], suggesting that in vitro expanded RSCs are distinct from retinal progenitor cells. In conclusion, it is currently unclear whether the mammalian retina contains a cell-type that can be expanded in vitro as a multipotent stem cell to generate photoreceptors and further detailed analysis of RSCs including their origin, expression profile, and differentiation capacity have to be performed to judge their potential for regenerative transplantation approaches.

8.4 Retinal Pigment Epithelial Cells as Progenitor Cells

8.4.1 RPE: Characteristics and Function

In the layered structure of the retina the RPE, a neuroepithelium-derived cellular monolayer, is prominently located on Bruch's membrane between the photoreceptor outer segments and the choriocapillaris. Together with the photoreceptor layer, it constitutes a functional unit that provides the transducing interface for visual perception and is therefore pivotal in the maintenance of visual function [56]. The RPE is also a metabolically complex and active cell layer that is important for cellular and extracellular local homeostasis [57].

Besides its convenient location nearby photoreceptors, RPE possesses two known properties which are crucial for their potential role as source for cell replacement in retinal degeneration: proliferation and plasticity. Under normal conditions RPE cells are quiescent cells but they can proliferate and migrate after being activated under disease conditions [58, 59]. RPE proliferation may result in RPE regeneration/wound healing [60] and/or retinal detachment by dedifferentiation of the RPE towards cell generating tractional forces [61]. This is characterized by loss of their epithelial morphology, acquisition of a mesenchymal cell-like phenotype, and a decrease in their synthetic capacity. This correlates with decreased expression of the epithelial marker cytokeratin 18, redistribution of the actin cytoskeleton, and de novo expression of α-SMA [62]. During the pathological developments, RPE cells undergo even epithelial to mesenchymal transition (EMT) which is caused by cytokines as TGFβ [63, 64].

Additionally, RPE secrete different growth factors like pigment epithelium-derived factor (PEDF), known to be involved in neuronal differentiation and survival of stem/progenitor cells [65–67]. Finally, retinal remodeling triggered by retinal degeneration involves, besides focal cell loss, RPE trans- or dedifferentiation and invasion of the retina. As RPE cells can apparently transform into fibroblast-like cells, many unidentified fusiform cells in advanced human degenerated retina specimens and animal retinal degeneration models may be remnant survivor RPE cells [68]. Furthermore, approximately 10% of RPE cells isolated from adult human retina exhibit "stem cell-like" properties and can re-enter the cell cycle once in culture [69]. All these data are the initiation points for a recently increasing number of studies about progenitor cell-like properties of the RPE.

8.4.2 Endogenous Repair in the Retina

In retinal development neuroretina and RPE develop from the same original structure, the optic vesicle. It is a highly polarized structure, both in the dorso-ventral and proximal-distal orientation. Different growth and transcription factors are

involved in this developmental process: MITF is downregulated in the future retina while PAX6 stays on; in the future RPE, where MITF becomes prominent, PAX6 fades away [57]. Despite differentiating into highly specialized cells, RPE cells are a well-established source of retinal regeneration following a neural retina injury, at least in amphibians [69, 70]. In these animals, RPE cells commonly undergo a loss of their epithelial characteristics, acquire multipotency, and start proliferation. To produce new neurons, the RPE cells dedifferentiate, lose melanin pigment granules, and undergo several cycles of cell division [71]. However, the ability of RPE cells to produce retinal neurons decreases as embryonic development proceeds [72]. Furthermore, the potency of such RPE-derived cells seems to be different between species [69, 70]. In adult *Xenopus laevis*, a population of RPE cells acquires multipotency while expressing Pax6, migrates onto the retinal vascular membrane, and eventually transdifferentiates into a neural retina. The remaining RPE cells along Bruch's membrane participate in reforming the RPE. On the other hand, in the adult newt, all RPE cells seem to be converted into multipotent cells, which eventually generate both a new neural retina and RPE with correct polarity. In mammals, the RPE seems to have a limited potency to differentiate into retinal cells except for the RPE cell itself. However, they may retain some plasticity beyond this point as stem cell-like properties of RPE have been recently described. Adult human RPE cells could be activated to a self-renewing cell that loses RPE markers, proliferates extensively, shows neural and mesenchymal potency, and can redifferentiate into stable cobblestone RPE monolayer in vitro. This multipotent RPE sub-population was termed RPE stem cells [69]. Stem/Progenitor-like cells are also found in the rodent retina and include the pigmented cells of the ciliary body as well as the pigmented cells of the iris and the RPE [4]. Stem cells are defined as clonogenic, self-renewing progenitor cells that can generate one or more specialized cell types [73]. Especially, their ability to differentiate into various functional cell types is the major value for use in regenerative medicine. If somatic cells give rise to other than their own progeny they will dedifferentiate followed by redifferentiation. One example is the platelet-derived growth factor (PDGF)—modulated dedifferentiation and myoid differentiation of RPE cells as an initial step of proliferative vitreoretinopathy. Thereby, the mitotically quiescent, hexagonal RPE dedifferentiate towards migrating, flattened cells without epithelial characteristics. Finally, these proliferating RPE cells transdifferentiate to myofibroblasts or mesenchymal-like cells [74]. Thereby, proteins associated with highly specialized functions of the RPE are downregulated, whereas differential expression of proteins related to cytoskeleton organization, cell shape, cell migration, and mediation of proliferative signal transduction is induced [75]. Although, this is merely an example for pathophysiological events, these results show the capabilities of the RPE and might give new possibilities for the activation and/or modulation of endogenous repair mechanisms in the degenerated retina. As discussed earlier, MCs are the main candidates for this scenario in the retina, but RPE have been in the focus as well. In the following paragraphs we will highlight some of the recent developments in this regard.

8.4.3 *Experimental Approaches*

In retinal degeneration RPE and/or photoreceptors are affected by the developing destructive events. Therefore, the replacement of these cell types is crucial for any restorative or regenerative intervention. Several in vitro approaches have been pursued in order to use RPE cells as a source for photoreceptors. Cultured chick RPE cells were transduced with genes previously identified as capable of inducing RPE-to-photoreceptor reprogramming in the chick system [76]. These authors report efficient generation of differentiating, photoreceptor-like neurons from RPE cells through reprogramming with *neurogenin*1 (*ngn1*). In propagated cultures, the majority of the cells began to differentiate towards photoreceptors. Similar reprogramming could be achieved by using cultured mammalian RPE cells [77]. Thereby, the results showed that human RPE cell lines and primary cultures of porcine and mouse RPE respond to gene-induced reprogramming by giving rise to photoreceptor-like cells. The process of transdifferentiation is also a specific one as human RPE-derived SCs produce neural and mesenchymal, but not liver progeny after treatment with differentiation medium [69].

In vitro reprogramming of RPE progeny to differentiate into photoreceptor neurons might be used in future cell replacement studies. However, several issues have still to be solved: (1) harvest of the original RPE cells; (2) safety of the transfection/virus, and (3) RPE-specific method. To circumvent these hurdles reprogrammed or transdifferentiated cells should be better established in situ. Photoreceptor-like cells were developed from the RPE experimentally manipulated to express a regulatory gene participating in transcriptional networks leading to photoreceptor genesis during retinal development [78]. Therefore, transgenic mice were generated with a DNA construct that would express neurogenin 1 from RPE bestrophin-1 promoter or neurogenin 3 from RPE65 promoter. The animals contained photoreceptor-like cells in the sub-retinal space expressing photoreceptor proteins and displayed morphologic similarities to photoreceptors. The RPE was also maintained in these eyes. The described responsiveness of primary RPE cells for genetic manipulation in situ enhances the feasibility of RPE-to-photoreceptor reprogramming for endogenous photoreceptor replacement.

8.5 Conclusion

Although a number of challenges must still be addressed, the potential of SC and/or progenitor cell-based regenerative medicine to treat a variety of chronic, traumatic, or degenerative diseases holds great promise. Cell populations for cell therapies include embryonic or ASCs, progenitor cells, and all reprogrammed progenitor cells in vivo and in vitro. The use of SCs for regeneration of retinal degenerations and several experimental approaches has successfully replaced damaged photoreceptors and RPE using endogenous and exogenous SCs. Therefore, stem cells have

the potential to significantly impact retinal regeneration. The study of progenitors and adult retinal stem cells in vitro and in vivo has led to a better understanding of retinal development and enabled methods to direct stem and progenitor cells to specific fates. These methods may ultimately lead to the development of strategies for retinal repair. Further improvements in regard to integration efficiencies and the directed generation of rod or cone photoreceptors besides their specific enrichment will be of outmost importance for developing cell-based strategies towards clinical applications aiming to treat retinal degenerative diseases. A combination with bioengineering and the use of additional cell sources and cell types (e.g., Müller cells, inducible pluripotent stem cells) may bear even greater promise. However, ethical and scientific issues have yet to be solved.

Acknowledgments Many thanks to Nicole Körber and Claudia Göhler for their help to create and realize the figures and to format the manuscript. The work with this manuscript was kindly supported by funding from the Deutsche Forschungsgemeinschaft (DFG) FZT 111, Center for Regenerative Therapies Dresden, Cluster of Excellence to MA, the Berne University Research Foundation to VE and the German Federal Ministry of Education and Research, BMBF, PtJ-Bio, 0315883 to MF.

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Chapter 9
Stem Cells and the Ocular Lens: Implications for Cataract Research and Therapy

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Contents

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A. Pébay (ed.), *Regenerative Biology of the Eye*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4939-0787-8_9,

Abbreviations

ARN	Age-related nuclear
BMP	Bone morphogenic protein
ESC	Embryonic stem cell
FGF	Fibroblast growth factor
IGF	Insulin-like growth factor
IOL	Intraocular lens
iPSC	Induced pluripotent stem cell
Nd:YAG	Neodymium-doped yttrium aluminium garnet
PCO	Posterior capsule opacification
PSC	Pluripotent stem cell

9.1 Introduction

The transparent, avascular and non-innervated ocular lens is suspended in the light path between the cornea and retina by the zonular fibres within the zonula ciliaris (also called the Zonule of Zinn; Fig. 9.1). The lens provides approximately 30 % of the eye's focussing power and, through the combined action of the ciliary muscle and zonular fibres, the lens provides all the accommodating ability of the eye, that is, the ability to change focus between near and far objects. These dual properties of transparency and accommodation cause the lens to play a vital role in the development of key motor and social functions that require good vision.

Lens function arises from the lens' unique shape, cellular arrangement and cellular composition. Vertebrate lenses including the human lens are biconvex tissues surrounded by a flexible basement membrane called the lens capsule (Fig. 9.1). Within this capsule an anterior monolayer of lens epithelial cells overlies a mass of peripheral differentiating fibre cells and a central mass of terminally differentiated lens fibres [1]. Lens growth occurs throughout life as equatorial epithelial cells coordinately differentiate into successive layers of lens fibre cells that elongate to surround the underlying central fibre cell mass (also call the 'lens nucleus'). The tightly packed nature of the lens fibre cells reduces light scattering, as does loss of all organelles during terminal lens fibre differentiation. The expression and accumulation of large amounts of various crystallin proteins, such as α-, β- and γ-crystallins, provide the lens with the refractive index required for transparency and focussing.

Modulation of the lens' shape via the ciliary muscle and zonular fibres, together with the inherent flexibility of the lens capsule, allows the lens to change focus between near and far objects (accommodation). While a number of theories have been offered to describe the mechanism of accommodation, the most widely accepted

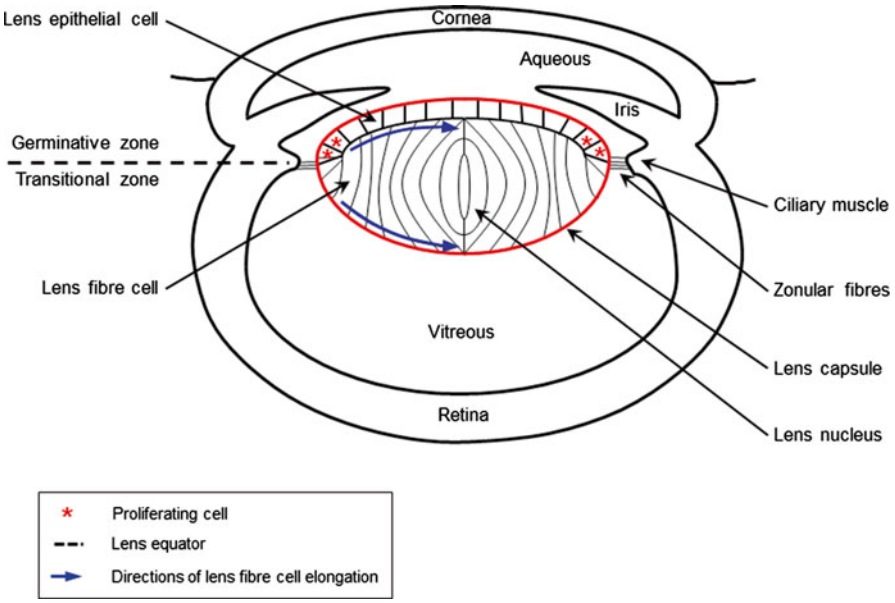

Fig. 9.1 Schematic diagram of the ocular lens within the eye

is that of Helmholtz [2, 3]. This theory states that when the ciliary muscle is relaxed, resting tension in the zonular fibres holds the lens in its least rounded (more flattened) shape, thus providing focus for distance vision. Upon accommodation for close vision the ciliary muscle contracts, causing the anterior ciliary body (to which the lens is attached via the zonules) to move toward the front of the eye. This reduces tension in the zonular fibres at the lens equator. Together with the elastic nature of the lens capsule, this movement causes the lens to take a more spherical form that enables close vision.

As a result of the lens' unique anatomy the aged lens contains embryonic lens fibre cells that have been exposed to environmental and light-induced insults throughout an organism's lifetime. Multiple protective systems such as UV filters and glutathione-based free radical scavenging have evolved to help delay the effects of these insults on lens function [4]. Nevertheless, the continuing increase in average human lifespan has meant that despite these protective systems diseases of the lens are becoming more prevalent worldwide, in particular presbyopia (loss of accommodation due to lens hardening) and cataract (loss of lens transparency). Some presbyopia and cataract treatments exist, however, they restore vision imperfectly and are costly due to the scale of these problems. Moreover, these treatments have unwanted side-effects such as the formation of secondary cataracts (also termed posterior capsule opacification or PCO), loss of accommodation, and life-altering visual disturbances such as glare and halos.

Recent studies suggest the formation of presbyopia and cataract may be linked [5–7]. Moreover, it has been estimated that a delay in primary cataract formation by 10 years could halve the need for cataract surgery [8–10]. While it is thought an improved understanding of the molecular mechanisms behind cataract formation will enable development of anti-cataract drugs [11], until now limited access to normal or cataractous human lens tissue has impeded anti-cataract drug development. The emergence of tissue-specific and pluripotent stem cell (PSC) technology [12–14] now offers an opportunity to generate large numbers of normal or diseased lens cells for research into the molecular mechanisms of presbyopia and cataract, as well as providing new hope for identifying drugs that inhibit or delay the onset or progression of these widespread diseases.

9.2 The Global Impact of Presbyopia and Cataract

Current estimates suggest that over a billion people worldwide are adversely affected by presbyopia, predominately in the developing world. Of those affected 410 million people are unable to perform near tasks as required [15]. Additionally, cataracts have caused over 80 million people worldwide to have low vision and almost 20 million people to be blind, thereby causing approximately 51 % of global blindness [16].

9.2.1 Prevalence and Causes of Presbyopia

Presbyopia begins affecting people from around age 40 and essentially all people over the age of 50 are affected [17]. As a consequence, most of the population in developed countries will spend about half their life dealing with the consequences of presbyopia-induced vision impairment, particularly difficulties performing activities close to the face. The development of presbyopia is most widely attributed to the observed loss of lens plasticity that occurs with age. Normally the centre of the lens changes shape during accommodation, yet as the lens ages its centre hardens [6, 18]. This hardening continues to the extent that human lenses over the age of 60 are incapable of changing shape in response to forces similar to those experienced by the lens in situ [19]. This increase in lens stiffness is thought to result from an accumulation of post-translational protein modifications within fibre cells at the centre of the lens [6]. These protein modifications are thought to result from decreased movement of protective lens substances into the centre of the lens, such as glutathione [4, 6, 20].

A controversial [2], alternate theory for the cause of presbyopia [21] has also been put forward based on the observations that the lens continues to grow throughout life via the addition of secondary lens fibre cells at the lens equator. This gradual increase in lens diameter has been proposed to gradually reduce the distance between the lens and the ciliary body, such that from around the age of 40 it may be sufficiently close to the ciliary body to reduce the effect of maximal stretch of the ciliary muscle.

This would then reduce the amount of force that can be applied by the ciliary muscle to change the lens' shape, thus reducing the focal range of the lens [21, 22].

9.2.2 Limitations of Current Presbyopia Treatments

The most common current treatment for presbyopia involves the use of spectacles for near vision. A combination of spectacles for near vision and other spectacles for distance vision may also be required, or alternately a single pair of bi-focal, multi-focal or progressive lensed spectacles may be used [23]. These approaches can provide good correction for both distance and near vision, though intermediate vision is often inadequate. Other inherent difficulties with spectacle use for presbyopia correction include discomfort; 'image jump' and the requirement to have a fixed gaze for clear near and distance vision (particularly relevant to bi- and multi-focal spectacles); and low utility [23]. For many patients the need to wear spectacles after a lifetime without them can necessitate workplace and recreational changes that require significant lifestyle and emotional adjustments. This is further compounded as people become more forgetful with age and find it difficult to locate and/or protect spectacles or, in some cases, decide to continue their normal lifestyle (e.g. driving) with impaired, uncorrected vision.

Contact lenses were naturally considered as an alternative to spectacle-based presbyopia correction. Contact lenses for monovision are used (i.e. one eye corrected for near vision and the other eye for distance) as are multi-focal contact lenses [24]. However, the use of contact lens for presbyopia treatment is not widespread [25, 26] and is often discontinued due to inherent glare and halos that affect routine daily activities (such as driving), or due to an inability to adapt to monovision. Other negative factors that impact on contact lens use for presbyopia correction include discomfort; dryness, particularly due to reduced tear film production and stability with age, decreased eyelid tone/strength with age, infection, on-going cost and difficulties in the daily close-handling required for use [23, 24, 27].

Currently no pharmacological intervention has been identified that inhibits or delays the onset or progression of presbyopia. Accordingly there is a widely recognised and growing need for new presbyopia treatments that improve or provide accommodation without the significant unwanted side-effects of spectacles and contact lenses. In an attempt to achieve this, various forms of 'refractive surgery' are being developed and trialled in the developed world. Surgically induced presbyopic monovision, through the use of different intraocular lenses (IOLs) in a patient's two eyes, is one approach being tested. Other approaches include corneal implants and laser-based corneal shaping [2, 27–29]. Multi-focal IOLs and accommodating IOLs are also being developed and/or tested (see Sect. 2.4). However, none of these methods have proven sufficiently successful to gain widespread use [2, 27–29]. Despite the controversy over the contribution of reduced ciliary muscle function to presbyopia formation, two scleral modification clinical trials have also been initiated: one testing scleral expansion bands and the other testing laser treatment of the sclera in an attempt to enable freer ciliary muscle movement [28, 30]. As yet no results from either trial have been published, however, data from other studies suggest that at least the scleral band approach will provide little if any long-term benefit [31–34].

9.2.3 Prevalence and Causes of Age-Related Cataracts

Cataracts, resulting from lens opacities that decrease lens transparency and increase light scatter, are the leading cause of low vision and blindness outside of uncorrected refractive errors [16]. As indicated above, over 100 million people worldwide have low vision or are blind due to cataracts. Cataracts are often considered a disease of ageing as the prevalence of the main forms of cataract increase with age all over the world [35]; this includes nuclear cataract, often termed age-related nuclear (ARN) cataract, cortical cataract and posterior subcapsular cataract. For example, the prevalence of ARN cataract has been estimated to increase in Australia from 0.4 % in people aged 50–54, through to 80.4 % in people aged 85 or older. The Beijing Eye Study estimated nuclear cataract prevalence to be 7 % in people aged 50–54, and 98.2 % in people aged 75 or older. Studies from other countries fall in or around these ranges and, while some variation is introduced due to the use of different cataract definitions and grading systems, it is clear that cataract prevalence increases with age.

Cataract is also a growing international problem due to the increasing average population age across the globe. Estimates suggest that between 2001 and 2021 the number of Australians over the age of 50 affected by cataract will increase by 63 %, yet the population is estimated to only increase by 19 % over this time [36]. Similar trends in other countries including America, Europe, India, Africa and China helped lead to the establishment of the World Health Organisation Vision 2020 program aimed at reducing the effects of global blindness [37, 38].

Of the three main types of age-related cataract, ARN cataract is generally the most common and accounts for 50 % of total cataract cases [35]. ARN cataract presents as partial or complete opacification of the lens nucleus that contains the embryonic and foetal lens fibre cells. Numerous studies have shown that ARN cataract is associated with darkening of the lens (brunescence) to yellow and then red and brown [5, 39], a process thought to involve chemical modification of lens proteins [4, 18]. A range of aberrant post-translational protein modifications have been described, all of which could contribute to light scatter within the eye; these include glycation, deamination and oxidation of crystallin proteins, as well as protein aggregation, crosslinking and insolubilisation. Ultrastructural analysis of lenses with ARN cataract also shows the appearance of multilamellar bodies that are likely to also contribute to light scatter within the lens [40–42].

A range of risk factors appear to promote ARN formation including older age, female gender (due to issues such as life expectancy and access to care), smoking, obesity, UV light, heat, oxidation and lower levels of education [4, 43, 44]. However, the molecular mechanisms of ARN cataract formation remain ill-defined. One current theory suggests that a diffusion barrier develops during lens ageing that inhibits diffusion of antioxidants (like glutathione) into the centre of the lens, thus leading to abnormal accumulation of protein modification, protein insolubilisation, and consequently light scatter [4, 20, 45].

In contrast to ARN cataract, age-related cortical cataracts occur in the peripheral (or cortical) lens fibre cells that surround the fibre cells within the lens nucleus. Cortical cataracts can present in a variety of morphologies including dot-like, radial,

circular and spoke-like opacities [5]. Mechanical stress between flexible cortical fibre cells and less flexible aged nuclear fibre cells is thought to contribute to cortical cataract formation. Altered intracellular Na^+, K^+ and Ca^{2+} ion concentrations may also contribute to cortical cataract through over-hydration, protein loss and proteolytic cleavage of structural proteins within fibre cells.

The least common type of age-related cataract is posterior subcapsular cataract [35]. This includes secondary cataract, or PCO, that results from the migration of residual lens epithelial cells along the posterior lens capsule. These epithelial cells then undergo aberrant fibre differentiation processes that, together with an associated wrinkling of the posterior capsule, causes light scatter [46]. Although the least common form of age-related cataract, PCO is still clinically relevant and costly to treat as it is a routine consequence of treating primary cataracts (see Sect. 2.5).

9.2.4 Limitations of Current Age-Related Cataract Treatments

At present there is no accepted pharmacological intervention available to inhibit or delay the formation of age-related cataracts. Some epidemiological data suggests that populations with reduced levels of vitamin C have higher levels of cataract [47], and that higher dietary intake of carotenoids and vitamin E decreases the risk of age-related cataract in women [48, 49]. However, randomised clinical trials of dietary or supplementary beta-carotene [50] or vitamin E in women [51] showed no decrease in the risk of age-related cataract. Similarly, long-term randomised trials of dietary supplementation with beta-carotene, vitamin C and/or vitamin E demonstrated no protection against age-related cataract for men or women [52, 53].

The inability to effectively inhibit or delay cataract formation or progression has meant that surgery is the only option for restoring vision in cataract patients. As a result, surgery for primary cataracts (particularly ARN cataracts) is the most commonly performed ophthalmic procedure worldwide. In Australia over 180,000 cataract operations are performed annually costing over $326 million [54–56]. In the United States, approximately three million cataract operations are performed annually at a direct cost of $6.8 billion [57]. The annual number and cost of primary cataract operations therefore places a massive financial burden on medical systems worldwide. Extensive waiting lists of visually impaired and blind patients can result, even in first-world countries, that decrease quality of life and increase stresses on patients and their families [58]. In third-world countries inadequate access to surgery leaves many patients blind.

In developed countries cataract surgery most often involves removal of the non-transparent cataractous lens while leaving the lens capsule in place (extracapsular cataract extraction). In third-world countries removal of the entire lens is often performed (intracapsular cataract extraction) to avoid the subsequent loss of vision due to development of PCO [59]. Once the aged cataractous lens is removed lens function is usually replaced through the implantation of a rigid, non-accommodating IOL or through the use of external, non-accommodating spectacles or contact lenses [59]. Unfortunately these approaches result in immediate loss of accommodation.

Attempts have been made to create multi-focal and accommodating IOLs for surgical treatment of both cataract and presbyopia, and a small number of these are commercially available. However, visual disturbances inherent to these IOLs such as glare, halos, reduced visual acuity and reduced contrast sensitivity result in these IOLs being unsuitable for many or most patients [60–63]. Careful patient screening is used to try and select patients likely able to cope with these disturbances, and extensive preoperative education is used to manage their expectations of postoperative vision. Nonetheless, these tools are imperfect and need improvement to ensure appropriate patient selection prior to implantation of the currently available multi-focal and accommodating IOLs [64]. Research into injectable polymers with appropriate refractive index for lens replacement is also being pursued, although this technology has not yet been translated into the clinic due to difficulties filling the lens capsule after cataract removal as well as the development of PCO [27].

9.2.5 Limitations of Current PCO Treatments

When access to primary cataract surgery is available various complications can arise including increased intraocular pressure, macular edema and PCO [59, 65]. In particular the inability to mechanically remove all lens cells during primary cataract surgery enables residual lens epithelial cells to migrate to the posterior capsule, resulting in PCO. A range of chemical approaches have been tried to remove these residual lens cells and avoid PCO formation [65], though even exposure of the residual cells to distilled water during primary cataract surgery has proven unsuccessful [66].

As a result of this inability to mechanically or chemically remove residual lens cells, a surgical approach is required to restore vision in patients with PCO. This method makes use of a neodymium-doped yttrium aluminium garnet (Nd:YAG) laser to perform a posterior capsulotomy. The piece of posterior capsule with attached cells that is cut away by the laser either peels back or falls away from the IOL to leave a clear light path [59].

Laser treatment of PCO has been estimated as the second most commonly performed ophthalmic procedure behind primary cataract surgery. In 1993 this treatment was estimated to cost $250 million annually in the United States alone [67]. The formation of PCO is affected by a range of factors including age at the time of primary cataract surgery (higher rates in younger patients), patient location (typically higher rates in developing countries), and time after cataract surgery (higher rates 3–10 years after primary cataract surgery). While the use of IOLs with sharp-edges has been reported to decrease or delay the rate of PCO, recent estimates indicate that PCO is still a significant burden. For example, the 10-year cumulative incidence of Nd:YAG laser treatment in Sweden was reported as 37 % for adults younger than 65 and 20 % for those over 65 [68]. A 10-year retrospective Austrian study showed similar rates [69]. Laser treatment for PCO also has its own side-effects including serious, vision-threatening complications such as retinal detachment reported to occur at a rate of ~0.4 to 4 % [70, 71]. Together, the high rate of PCO and the potential for severe

complications from Nd:YAG laser capsulotomy provide strong evidence of the need to develop new treatments for both PCO and primary cataract.

9.3 Lens Development and Cellular Architecture

The social and financial costs associated with treating presbyopia and cataract are increasing due to ageing of the world's population. However, it has been estimated that a delay in primary cataract formation by 10 years could halve the number of cataract surgeries needed [8–10]. Critically, the development of approaches to delay cataract formation is thought to require a more detailed understanding of the molecular mechanisms that drive lens, presbyopia and cataract development [4, 11]. Examining the role of stem cells in lens development offers an opportunity to understand cellular and molecular mechanism of lens formation, while potentially also providing cells for identification of candidate anti-presbyopia and anti-cataract drugs.

9.3.1 Embryonic Lens Development

The lens as a morphologically distinguishable tissue develops in the embryo from the lens placode, a dish-shaped thickening of the surface ectoderm on either side of the head closely opposed to the optic vesicles [1]. In most vertebrates the lens placode invaginates to form the lens pit which then separates from the surface epithelium to form a spherical monolayer of epithelial cells termed the lens vesicle. Under the influence of factors within the vitreous fluid and produced by the retina, cells of the posterior half of the lens vesicle initiate their fibre differentiation program, elongating toward the anterior epithelial monolayer to form primary lens fibre cells [1]. This process results in the lens vesicle lumen being filled with tightly packed elongated primary fibre cells. The process of lens fibre cell differentiation also consists of controlled organelle destruction that has similarities to apoptosis, including degradation of the endoplasmic reticulum and Golgi, as well as the mitochondria and the nucleus [72]. In this way objects larger than the wavelength of light that would otherwise cause light scatter are removed from the light path.

While these dramatic structural changes are occurring, large changes are also occurring to the protein expression profile within the differentiating lens fibres cells. This includes the expression of key cytoplasmic proteins that increase the refractive index of the lens (such as β- and γ-crystallins) as well as proteins that aid intercellular diffusion (connexins and aquaporins) within the terminally differentiated, avascular lens fibre cell mass.

During the process of lens vesicle production a thick basement membrane termed the lens capsule is produced that encapsulates the lens cells [73]. This transparent, smooth membrane assists with moulding the lens shape during accommodation, and contains collagen type IV, laminin, entactin, heparin sulphate proteoglycan and fibronectin synthesised by the lens epithelium [73].

9.3.2 Embryonic and Postnatal Lens Growth

After differentiation of the primary fibre cells forms the basic lens shape, the lens continues to grow throughout life while maintaining its biconvex shape and polarised epithelial and fibre cell arrangement. Coordinated differentiation of the lens epithelial cells along the equatorial edges of the anterior monolayer leads to development of the secondary lens fibre cells. These secondary fibres elongate along the anterior and posterior surfaces of the primary lens fibres to wrap them in consecutive layers of secondary fibre cells. As the lens continues to grow, the lens fibres in each successive secondary fibre cell layer become increasingly long as their apical and basal ends extend over the previously differentiated fibres (Fig. 9.1). Once the anterior and posterior poles are reached, the migrating fibre cell ends are precisely integrated with the neighbouring fibre cell ends to form the lens sutures [1, 74]. During an individual's lifetime, the progressive addition of new secondary fibre cell layers results in an increased density within the centre of the lens.

9.3.3 Lens Cell Characteristics Determine Lens Function

The coordinated lens fibre differentiation process leads to a precise fibre cell arrangement that is critical for transparency. The cross-sectional profile of the elongated fibre cells shows a highly ordered flattened hexagonal array where the spaces between each neighbouring cell are smaller than the wavelength of light, thus avoiding light scatter as light travels between the concentric fibre cell layers. Ultrastructural examination of the tightly packed fibre cell plasma membranes also shows specialised membrane interdigitations that interlock adjacent fibre cells in a manner thought to maintain the fibre cell alignment during accommodation [1, 75, 76].

The α-crystallin proteins produced by the lens epithelial cells, together with the α-, β- and γ-crystallin proteins produced by the lens fibre cells, help to increase the refractive index of the lens to that needed for focussing. Furthermore, the progressive production of β-crystallins early during differentiation and then γ-crystallins during terminal fibre differentiation creates a gradient of refractive index that helps to overcome aspects of spherical aberration which would otherwise degrade image quality [77]. Strikingly, the majority of these proteins are thought to not turn over within the post-mitotic, post-metabolic fibre cells and thus must remain stable for a lifetime in order to preserve lens transparency [78]. As mentioned previously, the loss of all organelles during terminal fibre cell differentiation removes these potentially light-scattering particles from the light path. In addition, the various fluid and solute transport mechanisms that operate within the lens enable the lens to be avascular, thus excluding light-scattering blood vessels from the light path [79].

9.4 Lens Stem Cells and Lens Regeneration

Together, the precise cell elongation, protein expression, organelle degradation, cell packing and solute transportation mechanisms that occur during lens formation and growth combine to enable the establishment and maintenance of lens transparency and accommodation. The fact that the lens can maintain its precise, critically required structure while continuing to grow successfully into adulthood strongly indicates the presence of a lens-specific stem cell population.

9.4.1 Evidence for Lens Stem Cells

To date, only a handful of studies have directly addressed the concept of a lens stem cell. In other organs, tissue-specific stem cells tend to be relatively rare populations of cells that have an extensive capacity for self-renewal, i.e. the ability to proliferate while maintaining the ability to differentiate into more mature cell types of the organs in which they reside. Stem cells in some systems, such as gut stem cells, proliferate more rapidly than stem cells in other systems, such as hematopoietic stem cells which tend to be predominantly quiescent.

A common feature of many tissue-specific stem cell systems is that they first produce highly proliferative immature cells termed progenitor or transit amplifying cells. These intermediate cells provide a burst of proliferation over a short period of time to increase the total number of cells available for differentiation into the tissue's more mature effector cells. By amplifying the effects of each stem cell division, these transit amplifying cells reduce the number of stem cell divisions required to maintain the production of a large number of terminally differentiated effector cells.

To define the numbers and locations of fast and slow cycling cells within the lens, a small number of studies have applied DNA-labelling techniques using tritiated thymidine and/or bromodeoxyuridine to mouse, rat, and chick lenses. These studies have shown that most proliferation occurs in the peripheral region of the anterior lens epithelium in the region known as the germinative zone (Fig. 9.1) [80–83]. These studies have also shown that cells in the central region of the anterior lens epithelial monolayer appear mostly to be mitotically quiescent. Longer term DNA-labelling experiments in mice (up to 18.5 weeks) have shown that cells within the central lens epithelium retained the largest amounts of label (i.e. have undergone the fewest cell divisions), and that these label-retaining cells can be induced to proliferate upon wounding [83]. In contrast, cells containing lower amounts of label at 18.5 weeks (i.e. cells that have undergone more cell divisions) were found in both the central and germinative zones, with some cells in the germinative zone also able to undergo proliferation after wounding [83]. Similar use of DNA staining, together with detection of the proliferating cell nuclear antigen, demonstrated that the most actively cycling lens epithelial cells are located in the germinative zone of mouse lenses, while the lens epithelial cells in the anterior region have a more dormant proliferative activity [84].

Together, these studies demonstrate that long-lived, relatively quiescent lens stem cells reside in the central lens epithelial monolayer that can be recruited to proliferation upon injury. More routinely proliferative stem cells and/or transit amplifying cells appear to reside in the germinative zone of the lens. Defining the growth factors and transcriptional apparatus that control the proliferation of these lens stem cells is of great importance for providing an understanding of the molecular mechanisms that drive the formation of presbyopia, primary cataract and PCO.

9.4.2 In Vivo Lens Regeneration from Lens Stem Cells

While only a few studies have explicitly tried to locate and characterise stem cells within the lens, the capacity for partial (though imperfect) in vivo mammalian lens regeneration after lens removal has been documented for almost two centuries [85]. From the first reported studies in rabbits, lens regeneration has been noted in a variety of other mammals including dogs, cows and rhesus monkeys.

Seminal work by Coulombre and Coulombre in the 1960s demonstrated that surgical reversal of embryonic chick lenses about the equator resulted in the formation of a new anterior epithelial monolayer from epithelial cells previously near the equator [86]. At the same time once the original epithelial monolayer was relocated to be in contact with the vitreous fluid, these lens epithelial cells differentiated into lens fibre cells in a manner reminiscent of normal embryonic primary fibre cell formation. A later study showed similar results using postnatal mouse lenses surgically transplanted into lentectomised adult mouse eyes [87]. These two studies demonstrated that: (1) all embryonic lens epithelial cells are capable of differentiating into lens fibres if provided with the appropriate stimuli; (2) the conditions necessary for the initiation and maintenance of lens fibre differentiation are provided by the vitreous fluid and (3) the conditions necessary for the growth and maintenance of the lens epithelial monolayer are provided by the aqueous fluid.

Even human lenses can show partial regeneration after primary cataract surgery in the form of Soemmering's Ring and Elschnig's pearls. Soemmering's ring forms when fusion of the anterior and posterior capsules traps proliferating and degenerating epithelial cells. In some cases, ultrastructural analysis has shown a degree of lens-like cellular organisation including a monolayer of epithelial cells on the residual anterior capsule, together with a mixture of fibre cells that can be arranged similar to the equatorial region of the normal lens [88, 89]. Additionally, Elschnig's pearls are transparent, globular masses of randomly mixed epithelial and fibre cells that appear after primary cataract surgery [89].

9.4.3 Identification of Lens Development Mechanisms Through Lens Stem Cell Research

Based on studies of in vivo lens regeneration, decades of research have been pursued in an attempt to discover the precise growth factors within the aqueous and vitreous fluids that are responsible for lens epithelial cell maintenance and lens

fibre cell differentiation. Research using chick lenses and explanted chick lens (stem) cells demonstrated that receptors for insulin and insulin-like growth factor (IGF) are expressed in these epithelial cells, and that insulin and IGF-I can stimulate elongation of chick lens epithelial cells in a manner reminiscent of lens fibre cell differentiation [90–92].

The establishment of rat lens epithelial (stem) cell explants demonstrated that one or more soluble factors from the neural retina can stimulate rat lens epithelial cells to undergo in vitro changes characteristic of lens fibre differentiation seen in vivo. This includes expression of β- and γ-crystallins, cell elongation and the development of specific plasma membrane interdigitations present in lens fibre cells in vivo [93–95]. Subsequent studies demonstrated that members of the fibroblast growth factor (FGF) family, particularly FGF1 and FGF2, can stimulate explanted rat lens epithelial cells to proliferate, migrate or differentiate into lens fibre cells depending on the FGF concentration used [96, 97]. In particular, high concentrations of FGF (100 ng/mL) stimulated the explanted rat lens epithelial cells to elongate, develop membrane interdigitations and express β- and γ-crystallins [96, 97].

This progressive cell behaviour in response to FGF signalling was noted as reminiscent of the progression of proliferation, migration and differentiation that occurs at the lens equator in vivo. To test the effect of FGFs on lens development forced FGF overexpression in mouse lenses was used [98–100]. This resulted in the accumulation of lens fibre cell characteristics within the lens epithelial cells. Characterisation of FGF receptor expression in mouse lenses [101, 102] led to targeted knockout of these receptors, thus demonstrating that FGF receptors are required for normal mouse lens fibre differentiation [103]. Finally, analysis of FGF concentrations in aqueous and vitreous fluids demonstrated higher FGF levels in vitreous compared to aqueous [104].

9.4.4 In Vitro Lens Regeneration from Lens Stem Cells

The weight of evidence implicating FGFs in lens development and growth, together with the higher FGF concentration in vitreous fluid compared to aqueous, led to the hypothesis that an increasing anterior-to-posterior FGF concentration gradient within the ocular fluids is responsible for establishing the basic lens structure [104–107]. Indeed, experiments using two modified rat lens stem cell culture systems demonstrated that vitreous fluid is able to stimulate the regeneration of transparent, lens-like tissue in vitro from lens epithelial cells taken from the central region of rat lens epithelial monolayers [108, 109]. Paired rat lens explants cultured with bovine vitreous for over 30 days regenerated into functional, physiologically sized lens-like tissues approximately the same size as newborn rat lenses. These tissues contained an anterior monolayer of lens epithelial cells in contact with a large, organised mass of elongated, parallel-aligned fibre cells that expressed β- and γ-crystallins (Fig. 9.2). The fibre cells also had complex membrane interdigitations and underwent organelle loss in a manner remarkably similar to that seen during terminal lens fibre differentiation in vivo. Curiously, continued culture of these in vitro lenses resulted in the appearance of an opacity containing multilamellar bodies that were ultrastructurally similar to those seen in human ARN cataract [108].

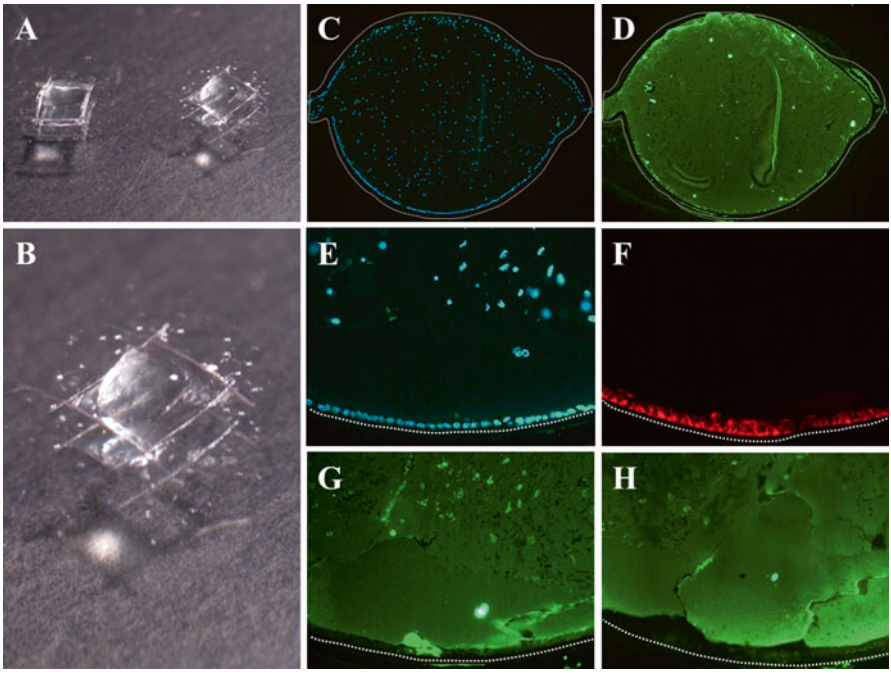

Fig. 9.2 Functional in vitro lenses generated from paired rat lens epithelial stem cell explants. (**a**, **b**) Photographs of cultured in vitro lenses focusing light. (**c–h**) Fluorescence images showing nuclei location within focusing in vitro lenses (**c**, **e**) as well as expression of α-crystallin (**d**) known to be expressed by lens epithelial and fibre cells, vimentin (**f**) known to be expressed by lens epithelial cells and β- and γ-crystallin ((**g**) and (**h**), respectively) known to be expressed by terminally differentiated lens fibre cells

Despite the key requirement shown for FGF signalling in lens development, using either FGF1 or FGF2 to replace the vitreous fluid within the paired rat lens stem cell cultures ablated the lens regeneration ability. Specifically, when FGF1 or FGF2 was used the paired rat lens stem cell explants underwent some changes characteristic of lens fibre differentiation; however, use of the individual FGFs resulted in the formation of non-transparent and incorrectly organised degenerating cell aggregates [109]. Thus, while FGF signalling appears to be a key requirement for lens development and growth, input from other growth factor pathways also appears to be required.

Investigation of insulin and IGF-I signalling using the traditional rat lens stem cell culture showed that, when applied individually, these factors induced some accumulation of β-crystallin within the lens cells [110]. However, neither γ-crystallin accumulation nor cell elongation occurred. In contrast, when applied in combination with FGF both insulin and IGF-I enhanced the fibre differentiation inducing effects of FGF, suggesting that the insulin/IGF pathway may yet prove to be important for lens development and growth. Whether additional signalling is also required from noggin, TGFβ/BMP, Wnt, PDGF, EGF and/or hedgehog pathways as some evidence suggests, or whether as yet undefined factors are required, remains to be determined [111, 112].

9.4.5 *In Vitro Lens Regeneration for Anti-Presbyopia and Anti-Cataract Research*

Overall, the demonstration that functional, correctly organised, but ultimately cataractous in vitro lenses can be produced from rat lens stem cells suggests these novel culture systems can provide important insights into the molecular mechanisms operating at all stages of ARN cataract, and possibly presbyopia, formation. Further investigation could define the molecular mechanisms of existing risk factors for cataract and presbyopia such as oxidative stress, heat or UV light. Alternately, new cataract and presbyopia risk factors might be identified. Importantly, these studies can be done more simply and in greatly reduced timeframes compared to collection and analysis of human patient cataract samples.

With the increasing incidence of presbyopia and age-related cataract the need for new treatments for these conditions is clear. Given the link between presbyopia and age-related cataract, strategies that inhibit or delay either of these two conditions could help to provide the 10-year delay in cataract formation needed to half the number of required cataract surgeries [8–10]. Thus the use of rat lens stem cell culture systems to create functional, but ultimately cataractous, in vitro lenses would appear to be an ideal tool to use in understanding the mechanisms of age-related cataract, and possibly presbyopia, development. These rat lens stem cell systems could also be used for discovery of anti-cataract and/or anti-presbyopia drugs; any treatment that delayed cataract formation in these in vitro rat lenses could have enormous public health cost savings and could greatly increase patient quality of life.

9.4.6 *In Vivo Lens Regeneration Via Transdifferentiation of Non-lens Cells*

In addition to the in vivo and in vitro evidence of partial mammalian (and avian) lens regeneration from lens stem cells, a large body of evidence has demonstrated lens regeneration can occur from non-lens cells in urodeles (newts and salamanders), Xenopus frogs and some fish [113, 114]. This process was first noted in urodeles during the 1890s by Collucci (1891) and separately by Wolff (1895) and is often referred to as Wolffian regeneration or transdifferentiation (meaning the de-differentiation of one cell type and its subsequent differentiation to another cell lineage).

Despite the use of similar terminology a variety of differences exist between lens regeneration in urodeles and frogs [114]. In newts, lens regeneration occurs from pigmented epithelial cells within the dorsal iris; in Xenopus frogs lens regeneration occurs from corneal epithelial cells. Additionally, urodeles are able to regenerate lenses repeatedly throughout life while in Xenopus lens regeneration can only occur before metamorphosis of the tadpole. Moreover, it is currently unclear whether Xenopus lens regeneration actually requires de-differentiation of the corneal epithelial cells, or whether the competence for both corneal and lens differentiation persists in these cells due to the incomplete differentiation status of the Xenopus corneal epithelium prior to metamorphosis.

For both urodeles and Xenopus species the anatomical changes that occur during lens regeneration have been well defined (as reviewed in detail by Henry and Tsonis) [114]. In newts lens removal stimulates proliferation of pigmented epithelial cells in the dorsal iris, followed by their de-differentiation (as seen by the loss of pigment) and formation of a lens vesicle-like structure. The subsequent differentiation of the posterior cells of this vesicle forms the primary fibres that elongate until they meet the anterior epithelial cells, thus filling the lumen of the newly formed lens. Secondary fibres are then added via differentiation of the epithelial cells at the lens equator. After Xenopus lens removal and associated damage to the corneal endothelium, secreted retinal factors are able to diffuse anteriorly to the inner layer of the corneal epithelium directly over the pupillary opening. These factors then stimulate lens regeneration via formation of a lens vesicle-like mass, followed by differentiation of the posterior cells into primary fibre cells, and addition of secondary fibre cells via differentiation of equatorial epithelial cells in the newly formed lens.

Interestingly, growth factors involved in normal embryonic lens development also appear to be involved during lens regeneration via transdifferentiation. In newts, FGFs and their receptors are expressed during lens regeneration and injection of FGF2 by itself can cause lens regeneration via transdifferentiation [115]. Furthermore, injection of a soluble recombinant form of FGF receptor 2 isoform IIIc inhibited newt lens regeneration [115], as did the FGF receptor inhibitor chemical SU5402 [116]. Understanding the molecular mechanisms that drive lens regeneration in urodeles and Xenopus might provide new information to better understand mammalian lens development. Curiously, newt lens transdifferentiation is also characterised by increased expression of some of the genes involved in production of induced pluripotent stem cells (iPSCs), including Sox2, Klf4 and c-myc but apparently not Oct4 or nanog [117]. Based on these observations it has been suggested that a molecular understanding of newt lens transdifferentiation might provide insight into the molecular mechanisms of somatic cell reprogramming.

9.5 Human PSCs and Lens Research

While important evolutionary-conserved mechanisms of lens development can be defined using animal models, known differences in lens development, structure and composition between species highlight the limitations of relying exclusively on animal models. For example, in transdifferentiation models of lens regeneration the dissimilarities to mammalian lens are obvious; the stem cells that enable lens regeneration are non-lens cells of the iris or cornea which may not completely replicate the biology of the human lens cells involved in the formation of presbyopia, primary cataract and PCO.

Even amongst vertebrates there are differences in lens development. In some vertebrate species including the chick, mouse, rat, rabbit, newt and humans, the thickened lens placode that invaginates from the surface ectoderm initially forms a lens vesicle with an acellular lumen [1, 118, 119]. In other vertebrate species, including the zebrafish and frog, the lens delaminates from the surface ectoderm as

a solid cluster of cells not as a vesicle [120–123]. Likewise, the final morphology of animal lenses can differ significantly between species, particularly around the alignment and structure of the apical and basal lens fibre cell tips located at the anterior and posterior lens sutures [74, 76]. Cross species differences also exist in crystallin expression: while α-, β- and γ-crystallins are found in all vertebrates there are also taxon-specific crystallins such as δ-crystallin in bird and reptile lenses, and ρ-crystallin in frogs lenses [124, 125]. Similarly, the refractive index in the periphery and centre of lenses differs between species.

Given these differences in lens embryology, structure, composition and function that exist between species, the development of new presbyopia and cataract treatments solely using animal models is prone to the risk that these treatments will not be reproducible in humans. The emergence of human PSC technology, including embryonic stem cells (ESCs) [12, 13] and iPSCs [14], offers an opportunity to circumvent this risk by providing a large-scale source of normal or diseased human lens cells for research [126] and drug screening [127].

9.5.1 Lens Differentiation Methods for PSCs: Progress and Problems

To date, three methods have been published that differentiate PSCs into lens cells: co-culture with mouse PA6 stromal cells [128]; sequential addition of recombinant growth factors aimed at mimicking embryonic lens development in vitro [129]); and use of chemically defined, serum-free medium together with cell purification via flow cytometry [130].

Mouse ESCs cultured on a monolayer of PA6 stromal cells have been shown to support the induction of eye-like structures that contain cells expressing specific phenotypic markers of lens cells (αA-, αB- and β-crystallins) and pigmented retinal cells (Brn3b, syntaxin) [128]. The gross morphology of these structures showed most of the ocular cell types, except retinal pigment epithelium, were mixed within the multilayered cell masses with no organised structure apparent. While this study showed that lens cells could be produced from PSCs it did not define the factors responsible for the induction of the eye-like structures (though it did determine that FGF2 alone was insufficient).

The first report of human PSC differentiation to lens cells was published in 2010 [129]. This study described a three-stage growth factor treatment that differentiated human ESCs first into neuroectoderm (via Noggin), lens progenitor cells (via FGF and BMPs) and finally into lens epithelial and fibre cells (via FGF and Wnt). While lens cells were produced via this method, non-lens cells were also produced. The lens cells that were present were also inappropriately organised within three-dimensional lens-like structures called ‘lentoids’ that expressed α-, β- and γ-crystallins.

In an attempt to obtain a population of purified lens epithelial cells, human ESCs were more recently cultured in a chemically defined, serum-free medium supplemented with selenium and human recombinant insulin and transferrin [130]. As this

differentiation method resulted in a heterogeneous population of neural ectodermal, non-neural ectodermal and mesodermal cells, a trial-and-error fluorescence-activated cell sorting approach was used to try and purify the lens epithelial-like cells. Based on literature reports of lens cell protein expression, a highly complex and non-scalable separation method was obtained. This method involved targeted selection of lens cells that expressed c-Met and/or CD44, with simultaneous depletion of cells that expressed p75, HNK-1 and CD15. The c-Met+, c-Met+/CD44+ and CD44+ positive populations contained lens-like cells, though with non-lens cells present (particularly the CD44+/c-Met- cells). These sorted cell populations also spontaneously generated lentoid bodies that, similar to the lentoids seen with the sequential growth factor lens differentiation method, contained randomly arranged cells that expressed α- and β-crystallins. This c-Met/CD44-based approach was also highly inefficient, with only 0. 2 to 1.5 % of the total cells positive for these markers, while requiring the use of complex and labour-intensive multi-laser flow cytometry.

Based on current approaches, the ability to use human PSCs to investigate the molecular mechanisms that drive lens, presbyopia and cataract formation is limited by an inability to produce large quantities of purified lens cells. A simple, robust and scalable method for producing large numbers of purified lens epithelial cells from human PSCs needs to be established. Such a method would provide a cell source more relevant to the human condition than possible with animal models of lens, presbyopia and cataract formation.

9.5.2 Human PSC-Derived Lens Cells for Identification of Developmental Mechanisms

Access to large numbers of human PSC-derived lens cells would offer an opportunity to define molecular mechanisms of lens development, lens epithelial cell maintenance and lens fibre cell differentiation without the concern of identifying species-specific events. The derivation of human iPSCs from patients affected by congenital cataract would also enable elucidation of molecular mechanisms that cause congenital cataracts. Importantly, purified human PSC-derived lens cells derived from normal or diseased human PSCs would offer an opportunity to better understand the mechanisms of cataract formation, as will likely be needed to develop anti-cataract drugs [11].

The lens has also long been used as a model of development and differentiation for other human tissues [131]. Molecular insights of lens development obtained from human PSC-derived lens cells would therefore provide a broader understanding of cellular processes applicable to other organs. This would likely include control mechanisms of proliferation, migration and cell packing, as well as mechanisms of differentiation and apoptosis. Mounting evidence also indicates that age-related cataract, particularly ARN cataract, is an independent predictor of mortality (as recently reviewed by Gower and West) [35]. Thus an understanding of the

molecular mechanisms of cataract formation provided by human PSC-derived lens cells may provide more broadly applicable insights into the mechanisms of systemic ageing and premature death.

9.5.3 Human PSC-Derived Lens Cells to Identify Anti-PCO Drugs

The advent of quantitative high-throughput chemical screening methods within the pharmaceutical industry has provided the means for rapid novel drug discovery. While a human in vitro capsular bag model has been used to test for anti-PCO drugs [132], this method is not suitable or scalable for high-throughput drug screening. In contrast, purified populations of human PSC-derived lens epithelial cells would, for the first time, provide an opportunity for high-throughput screening to identify candidate anti-PCO drugs. Significant public health savings and improved primary cataract surgery outcomes in both the developed and the developing world, could be realised if such agents, applied either at the time of primary cataract surgery or via slow release IOLs [133], could replace the current need for PCO treatment via Nd:YAG laser posterior capsulotomy. The derivation of human iPSCs that contain cataract-causing mutations would also enable the development of powerful new 'disease in a dish' models to better understand the developmental origins of primary cataracts.

Lens toxicology screening systems have also been proposed to study the mechanism of drug-induced cataractogenesis, based on cultured rat lens stem cell explants [134]. Similarly, access to large numbers of purified human PSC-derived lens epithelial cells would enable the development of new toxicity assays that could streamline ocular drug development.

9.6 Summary

Presbyopia and cataract are large, expensive and increasing global problems that begin affecting all people who live beyond 40 years of age. Due to global population ageing, new methods of prevention and treatment are needed to cope with the projected increase in these diseases, and also to improve treatment outcomes in ways that could provide massive economic benefit.

Valuable information relating to evolutionary-conserved molecular mechanisms of lens, presbyopia and cataract development will continue to be gained through the use of animal models. The use of primary rat lens stem cell cultures to regenerate clear, then ultimately cataractous, in vitro lenses is of particular interest. Understanding the molecular mechanisms that drive these processes will have major implications for the development of anti-cataract and possibly anti-presbyopia

therapies. Similarly, the establishment of a simple, robust and efficient method for generating purified lens epithelial cells from human PSCs will provide much-needed new avenues for understanding lens, presbyopia and cataract formation as well as providing a new tool for novel drug discovery.

Thus while key technical challenges need to be resolved, tissue-specific and human PSC-derived lens cells offer an exciting and real opportunity to identify and develop new and improved treatments for presbyopia and cataract. Given the association between cataracts and early mortality, the information gained through these studies will also more broadly increase our molecular knowledge of the ageing process and associated diseases.

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Chapter 10
Trabecular Meshwork Stem Cells

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Contents

Abbreviations

ABCG2	ATP-binding cassette transporter G family member 2
AQP1	Aquaporin 1
BrdU	Bromodeoxyuridine
CHI3L1	Chitinase 3-like 1
DMEM	Dulbecco's modified eagle's medium
DMEM/F12	Dulbecco's modified eagle's medium/nutrient mixture F-12
FACS	Fluorescence activated cell sorting

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A. Pébay (ed.), *Regenerative Biology of the Eye*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4939-0787-8_10,

FBS	Fetal bovine serum
IOP	Intraocular pressure
JCT	Juxtacanalicular connective tissue
MGP	Matrix Gla protein
POAG	Primary open angle glaucoma
SC	Schlemm's canal
SCGM	Stem cell growth medium
SP	Side population
TM	Trabecular meshwork
TMSC	Trabecular meshwork stem cells

10.1 Introduction

10.1.1 Aim of this Chapter

Glaucoma is a leading cause of irreversible blindness worldwide and the second leading cause of blindness overall in the United States [1]. Glaucoma is a progressive optic neuropathy with loss of retinal ganglion cells and optic nerve axons, resulting in visual field impairment.

Elevated IOP and aging are important risk factors for most forms of glaucoma including primary open angle glaucoma (POAG). Pathological changes in the TM and Schlemm's canal endothelium are prime suspects for increased resistance to the aqueous outflow and elevated IOP. It has been suggested that age and disease-related decrease of TM cells [2–6], abnormal accumulation of extracellular matrix (ECM), and appearance of cross-linked actin networks in the TM cells [7–9] contribute to increased resistance of the aqueous outflow and subsequent increase of IOP. Although the pathogenesis is multifactorial, optic nerve damage is strongly associated with increased IOP. Experimental animal models demonstrate that elevated IOP is sufficient to produce glaucoma-like optic nerve damage [10].

Current therapies for IOP control involve pharmacologic reduction of aqueous humor production and surgical or pharmacologic enhancement of outflow. These therapies are effective but have significant limitations; toxicity, side effects, complications, failure, and patient noncompliance are common. Resident pools of somatic stem cells in many organs are responsible for tissue maintenance and repair. Many of these stem cells expanded in vitro exhibit effective tissue regeneration when introduced to pathologic tissues in vivo [11]. Stem cells from trabecular meshwork may have a potential for development of a novel cell-based therapy for glaucoma.

This chapter will review literatures and describe the methods used in identification, isolation, and culture of trabecular meshwork stem cells (TMSCs) and introduce the characteristics of TMSCs. The potential for using these stem cells in therapeutic applications in glaucoma treatment will be discussed.

10.1.2 Structure and Cells of the Trabecular Meshwork

Trabecular meshwork (TM), together with the juxtacanalicular connective tissue (JCT), the endothelial lining of Schlemm's canal (SC), the collection channels, and the aqueous veins comprise the conventional or trabecular outflow pathways. The TM outflow pathways provide resistance to aqueous humor and at the same time allow bulk flow of aqueous humor pass through it by the IOP gradient, thus keeping IOP in a steady state. Outflow resistance in the TM outflow pathways increases with age [12, 13] and in primary open-angle glaucomatous eyes [14, 15].

The TM occupies most of the inner aspects of the sclera sulcus, while the SC lies in the outer portion of it. The TM is a porous filter-like structure formed by connective tissue beams of lamellae that have a core of collagenous and elastic fibers that are covered by flat cells. The TM consists of the inner uveal meshwork, the deeper corneoscleral meshwork, and JCT that is localized directly adjacent to the inner wall endothelium of SC [16]. The uveal and corneoscleral parts of the TM do not provide a significant resistance to aqueous humor outflow [17, 18], whereas the JCT and SC inner wall endothelium maintain the main resistance to aqueous outflow [19, 20]. The TM is divided to a filter portion and a nonfilter portion by whether the tissue is around the SC or not. The nonfiltering portion of the TM which resides beneath the Schwalbe's line is believed to harbor a niche for cells with adult stem cell-/progenitor cell-like properties that are capable of dividing and repopulating the filtering part of the TM after injury [21].

The cells lining the lamellae of the TM play two primary roles: secretion of specific enzymes and ECM and phagocytosis of debris in the aqueous humor [22]. Both functions help maintain aqueous outflow over the trabecular lamellae [23]. TM cells also release ligands that regulate permeability of SC endothelial cells to regulate transendothelial flow [24]. Reduced cellularity within the TM is observed with age and correlates with increased outflow resistance and elevated IOP [2, 4–6]. TM cells play an important role in regulating outflow facility.

10.1.3 Stem Cells in the Trabecular Meshwork

Many researchers have reported studies related to stem cells of trabecular meshwork but identification and characterization of putative TMSCs is currently incomplete. In 1982, Raviola [25] identified an unusual cell population termed Schwalbe's line cells with distinct ultrastructural features different from TM cells. These cells form a discontinuous cord, oriented circumferentially at the corneal periphery, deep to the endothelial lining of the anterior chamber [25]. In 1989, Acott et al. [26] reported an increased cell division of this cell population after laser trabeculoplasty in human corneoscleral explant organ cultures. The dividing cells migrate and repopulate the laser burn sites over the next few weeks [26].

Recently, Gonzalez et al. [27] cultured human TM cells as free-floating spheres and found that these spheres could be grown for more than 3 months expressing neural precursor marker nestin, as well as leukemia inhibitor factor. They concluded that the spheres may contain relatively undifferentiated cells derived from human TM. More direct evidence for the existence of stem cells in the TM was from an immunostaining study by McGowan et al. [28], demonstrating that some stem cell markers, such as nestin, alkaline phosphatase, telomerase, Oct-3/4, and Wnt-1, were found in the TM and in the transition zone between the TM and the corneal endothelial periphery.

Later on, a review article by Kelley et al. [21] showed that cells from TM insert region expressed HMFG-1 but not YKL-40 (also known as CHI3L1). The authors concluded that in the TM, the putative stem cells investigated as the Schwalbe's line cells may be the undifferentiated cell type. Yu et al. [29] did sphere culture on primary peripheral bovine corneal endothelial cells; those spheres expressed nestin. When the spheres were induced for differentiation, they were positive to neuronal marker β-III tubulin. Thus the authors hypothesized that the cells in the transition area between the corneal endothelium and TM may be progenitors for both corneal endothelial cells and TM cells.

In 2012, we reported the results of isolation of TMSCs from human TM tissue by side population cell sorting and by clonal culture and of characterization of these cells [30]. The TMSCs have distinct properties from primary TM cells. They are multipotent and can differentiate into phagocytic TM cells [30]. Tay et al. [31] reported the presence of mesenchymal stem cells in human TM which expressed CD73, CD90, and CD105. To further study the ability of the TMSCs in vivo, we injected human TMSCs into normal mouse anterior chamber and detected that the TMSCs can home to the TM region and maintain stem cell characteristics or become functional TM cells without causing IOP elevation [32]. These stem cells present a potential for development of a novel cell-based therapy for glaucoma.

More recently, Nadri et al. [33] reported isolation of mesenchymal stem cells from the TM and the cells can be induced to differentiate into photoreceptor-like cells on amniotic membrane. This broadens the possible clinical applications of stem cells from the TM.

10.2 Isolation and Cultivation of Human Trabecular Meshwork Stem Cells

There are different published methods for isolation and cultivation of stem cells from the TM.

Gonzalez et al. [27] isolated and characterized free-floating spheres from human TM cell primary cultures. Primary TM cells were isolated as described by Stamer et al. [23] and cultured in low glaucoma Dulbecco's modified eagle medium (DMEM) with l-glutamine and 110 mg/L sodium pyruvate containing 10 % fetal bovine serum (FBS), 100 μM nonessential amino acids, and antibiotics at 37 ˚C in a humidified atmosphere of 5 % CO_2. Free-floating spheres were maintained in StemSpam™ Serum-Free Expansion Medium (StemCell Technologies, Seattle, WA).

Tay et al. [31] isolated TM cells following the method described by Tripathi and Tripathi [34] and digested the TM tissue with 2 mg/mL type I collagenase in DMEM containing 10 % FBS. Cells were cultured and passaged in low glucose DMEM containing 10 % FBS, 4 mM l-GlutaMAX™, 1 mM sodium pyruvate, 1 % nonessential amino acids and antibiotics.

Nadri et al. [33] cultured the cells in low glaucoma DMEM supplemented with 20 % serum and 200 ng/mL basic-FGF.

We [30] cultured the stem cells from the TM in stem cell growth medium (SCGM) modified from a corneal endothelial cell culture medium [35] containing multipurpose reduced-serum media (OptiMEM-1; Invitrogen, Carlsbad, CA) supplemented with 5 % FBS (Hyclone, Logan, UT), 10 ng/mL EGF (Upstate Biotechnologies, Billerica, MA), 100 μg/mL bovine pituitary extract (Biomedical Technologies, Stoughton, MA), 20 μg/mL ascorbic acid, 200 μg/mL calcium chloride, 0.08 % chondroitin sulfate, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL gentamicin (Sigma-Aldrich, St. Louis, MO).

We [30] used a standardized method, fluorescence-activated cell sorting (FACS), to isolate purified stem cells from primary cultured TM cells. Side population (SP) cell sorting has been used to isolate adult stem cells since the method was discovered in 1996 [36]. Either Hoechst 33342 dye [36] or DyeCycle Violet (DCV) dye [37] (Life Technologies) is carried out as previously described [30, 38]. After 2–3 passages, 5×10^5–2×10^6 cells are incubated at 1×10^6 cells/mL in prewarmed DMEM with 2 % FBS and 5 μg/mL Hoechst 33342 or 10 μM DCV for 100 min at 37 °C. A total of 1×10^5–5×10^5 cells are preincubated with 25 μg/mL fumitremorgin C for 20 min before Hoechst or DCV incubation to inhibit Hoechst or DCV dye efflux. After staining, cells are washed twice in Hank's balanced salt solution with 2 % FBS and stored on ice, then 2 μg/mL propidium iodide is added to identify nonviable cells immediately before sorting. Cells are analyzed on a flow cytometer high-speed cell sorter (FACSAria; BD Biosciences, San Jose, CA), using 350-nm (Hoechst) or 405-nm (DCV) excitation. Designated SP cells show reduced fluorescence at both blue (450 nm) and red (>620 nm) (Fig. 10.1).

10.3 Characterization of TMSCs

Gonzalez et al. [27] have shown that free-floating spheres could be grown for more than 3 months. These spheres can be promoted to attach to the substrate and cells could migrate out after addition of serum.

Kelley et al. showed [21] that TM insert cells had more expression on HMFG-1 and less expression on YKL-40 than mature corneal endothelial cells and TM cells.

Tay et al. [31] named the cells they cultured from TM as mesenchymal stem cells (TM-MSCs) since the cells expressed CD73, CD90, CD105, as well as ABCG2, Ankyrin3, LDLR, CHI3L1, HMFG-1, MMP1, and AQP1. The cells had clonal forming ability and could differentiate into adipocytes, osteocytes, and chondrocytes in vitro.

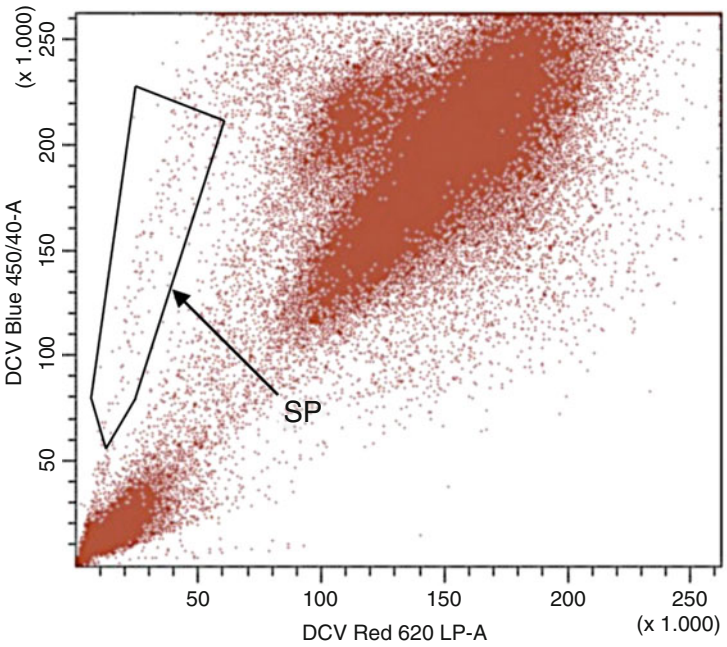

Fig. 10.1 Isolation of TMSCs as side population (SP) cells. SP cells were isolated by FACS from passage 3 human TM cells (using DyeCycle Violet Dye, Invitrogen). Cells showing reduction of both *blue* (450 nm) and *red* (>620 nm) are the SP cells in the frame. *FACS* fluorescence activated cell sorting, *LP* long pass. Reproduced from ref. [30] (Du et al.) with permission of the Association for Research in Vision and Ophthalmology

A group in Iran [33] also named the cells they cultured from TM as mesenchymal stem cells. These cells could be cultured as clones and could be induced to become photoreceptor-like cells expressing photoreceptor markers rhodopsin, PKC, and CRX.

Our group [30] isolated stem cells from the TM (TMSCs) by side population cell sorting or by clonal culture. The cells have distinct characteristics from primary TM cells. The TMSCs expressed stem cell markers OCT-3/4, ABCG2, MUC1 (also called HMFG-1), AnkG but not the TM cell markers CHI3L1, AQP1, MGP. The cell marker expression of TMSCs was detected by real-time PCR, immunofluorescent staining (Fig. 10.2) and immunoblotting.

One of the characteristics of adult stem cells is multipotent. We successfully induced TMSCs to differentiate into neuronal cells, adipocytes, corneal stromal keratocytes, as well as TM cells. Induced TM cells had similar gene expression profile to primary TM cells and were phagocytic, the same as primary TM cells.

One intrinsic property of adult stem cells is to identify and localize in specific tissues where they exhibit tissue-specific differentiation [39–42]. To test the homing ability of TMSCs in vivo, we injected human TMSCs into normal mouse anterior chamber and discovered that injected TMSCs had the ability to home to the TM region and become differentiated TM cells without damage to the TM and without IOP elevation [32].

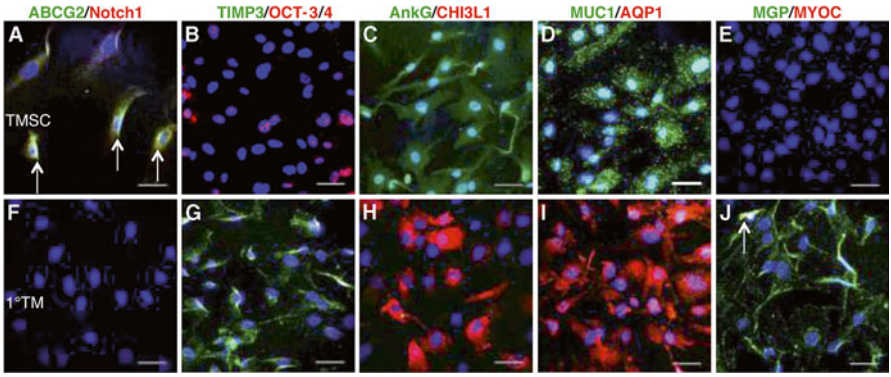

Fig. 10.2 Distinct gene expression profile of TMSCs from primary (1°) TM cells. Clonal passaged TMSCs (**a**–**e**) and 1° TM cells (**f**–**j**) were double stained with stem cell markers ABCG2 (*green*), Notch1 (*red*), OCT-3/4 (*red*), AnkG (*green*), MUC1 (*green*); TM markers TIMP3 (*green*), CHI3L1 (*red*), AQP1 (*red*), MGP (*green*); and MYOC (*red*). *Arrows* in (**a**) point to the ABCG2 and Notch1 double-positive cells. *Arrow* in (**j**) points to the MGP and MYOC double-positive cell. DAPI stains nuclei blue. Bars: 50 μm. Reproduced from ref. [30] (Du et al.) with permission of the Association for Research in Vision and Ophthalmology

10.4 Discussion

10.4.1 TMSCs Expresses Stem Cell Markers In Vitro

The presence of a stem cell population in the TM is confirmed in our studies on TMSCs [30, 32]. In vitro culture, TMSCs present a homogeneous population displaying antigenic markers previously characterized for mesenchymal stem cells (ABCG2, CD73, CD90, CD166, and Bmi1) as well as expressing gene products associated with pluripotent stem cells (Notch1, OCT-3/4). The stem cell markers that TMSC expressed, such as PAX6, MUC1, and AnkG, distinguish TMSCs from typical mesenchymal stem cells. PAX6 is a homeobox gene essential to ocular development and is present in some adult ocular tissues but not generally present in TM [43]. PAX6 is present in corneal stromal stem cells [38, 44] but not expressed by mesenchymal stem cells which come from the vasculature, bone marrow, or other tissues [45]. MUC1 is a cell surface mucin associated with breast and other epithelial cancers [46]. AnkG was recently described as essential for production of new neurons in the brain [47] and was described with higher expression in Schwalbe's cells that have been postulated to be responsible for cell regeneration in the TM [48]. The expression of these three genes in the TMSCs thus defines markers distinguishing these cells from bone marrow-derived mesenchymal stem cells.

10.4.2 TMSCs Differentiate into TM Cells in vitro

We have proved that TMSCs have multipotency with the ability to be induced to display phenotypic properties of cells from several different developmental lineages (neural, adipose, cornea) under specific culture conditions [30]. These cells are capable of differentiating into TM cells with phagocytic function and expressing TM cell markers AQP1, MGP, CHI3L1, and TIMP3 in the presence of aqueous humor or 10 % serum. The water channel aquaporin 1 (AQP1) has been detected in the TM in vivo [49] as well as in cultured human TM cells and plays an important role in modulation of aqueous outflow [23]. Matrix Gla protein (MGP) has the ability to function in the TM as a calcification inhibitor [50] and may be a key contributor to IOP homeostasis by regulating calcification and hardening of the TM [51]. Aqueous humor contains chitinase 3-like 1 (CHI3L1), which has a protective role against inflammation, ECM remodeling, and cell death in the outflow pathway [52]. All these support the hypotheses that these cells represent a resident population of adult stem cells in the human TM and differentiation to TM cells is the default lineage for these cells [30].

After induction, TMSCs also express TM proteins which have essential roles in TM function such as maintaining aqueous outflow.

10.4.3 TMSCs Preferentially Home to the TM Region After In Vivo Transplantation

Adult stem cells have an intrinsic property to identify and localize into specific tissues. Our experiments show that after xenotransplantation of human TMSCs into mouse anterior chambers, TMSCs preferentially localize to the TM region and maintain viability for at least 4 months [32]. The behavior of TMSCs in the anterior chamber is clearly distinct from that of corneal fibroblasts and has all the aspects of a classic homing response typical of adult stem cells. We believe that TMSCs were not simply being carried passively to the TM by aqueous outflow, but rather a result of a tissue affinity of the TMSCs. Increasingly abundant evidence supports the ability of mesenchymal stem cells to localize and regenerate damaged tissue in vivo [10, 53]. We thus hypothesize that in glaucomatous eyes, injected TMSCs may be able to localize to pathological TM and improve aqueous outflow. In glaucomatous eyes, abnormal extracellular matrix of the TM may have effects on stem cells' homing.

10.4.4 TMSCs Integrate into the TM Without Eliciting Inflammatory Response

Mesenchymal-like stem cells have been shown to possess the ability to mediate immunosuppression [4, 54–57]. Human TMSCs have the same capability of not eliciting inflammatory response after xenotransplantation to mouse anterior

chamber. It ensures the survival of transplanted stem cells to function in vivo. This observation provides an argument that these stem cells could be tolerated in human allogeneic transplantation. The ability of TMSCs to undergo extensive expansion in vitro makes allogeneic transplantation possible. Since glaucoma has underlying genetic components, it would not be feasible to do autologous transplantation using the same genetically abnormal cells. The expansion ability of TMSCs provides a possibility to regenerate TM in glaucomatous eyes by allogeneic transplantation of TMSCs without glaucomatous genetic disorders. Xenotransplantation of TMSCs also does not stimulate endogenous TM cell division. The endogenous TM cells in the eyes with the transplanted TMSCs were quiescent with no BrdU incorporation [32]. The injected cells do not affect the corneal transparency and do not cause increased IOP dramatically.

10.4.5 Possible Applications of TMSCs

We hypothesize that the TM is a self-renewing tissue maintained by a resident population of stem cells. This is supported by a recent study that has shown the TM cells expressed cell cycle and proliferation related genes [56]. To elucidate factors controlling TMSC proliferation in vivo might provide pharmacological approaches focusing on reconstruction of TM tissue and outflow pathway to control intraocular pressure.

Another exciting potential application of TMSCs is developing cell-based therapy for glaucoma. The ability of TMSCs to home to the TM and adopt a TM phenotype supports the idea that the TM in eyes with high IOP may be restored via such an approach. With the ability to alter the cellular composition and extracellular matrix of the TM, it will be possible to investigate the mechanism by which aqueous outflow is controlled by the TM cells. Information revealed by such studies can point the way to design a cell based-therapy approach to regulate aqueous outflow through the TM.

Acknowledgement The work was supported by an anonymous philanthropic donation to YD, National Institutes of Health Grant P30-EY008098, Eye and Ear Foundation (Pittsburgh, PA) and Research to Prevent Blindness.

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Chapter 11
Stem Cells of the Human Corneoscleral Niche

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A. Pébay (ed.), *Regenerative Biology of the Eye*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4939-0787-8_11,

Abbreviations

BM	Bowmans membrane
BMP	Bone morphogenic protein
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
iPS	Induced pluripotent stem (cell)
KSFM	Keratinocyte serum free media
LESC	Limbal epithelial stem cell
LiNS	Limbal neurosphere
LiPSC	Limbal induced pluripotent stem cell
LMSC	Limbal mesenchymal stem cell
LSCD	Limbal stem cell deficiency
mES	Mouse embryonic stem (cell)
MSC	Mesenchymal stem cell
TAC	Transit amplifying cell

11.1 Introduction

The ocular limbus is an accessible source of autologous stem cells that can be isolated, cultured, and transplanted for the treatment of limbal stem cell deficiency [1–3]. In this chapter we provide an overview of the cellular anatomy of the ocular limbus with a focus on the stem cells responsible for tissue maintenance and regeneration.

Primary limbal cell suspensions may contain a number of different stem or progenitor cell types that can proliferate in vitro. Therefore, current methods for limbal cell culture are reviewed and the cellular composition of primary limbal cell cultures is discussed. In addition, we examine claims of trans- and de-differentiation potential in limbal cell cultures. Limbal stem cells have been reported to display significant plasticity in response to growth factors and culture conditions, with the potential for neural lineage induction [4], neuronal [5] and photoreceptor differentiation [6], and even complete dedifferentiation into a pluripotent state [7–9]. However, although a number of laboratories have demonstrated neural lineage induction in limbal cell suspensions, it remains unclear which stem cells are contributing to this phenomenon.

The development of limbal stem cell culture systems [10] has enabled the implementation of stem cell replacement therapies for reconstruction of the damaged or diseased corneal surface [1–3]. Since limbal stem cells can be harvested in small biopsies from the surface of the eye with minimal risk or discomfort to the patient, they represent an accessible source of autologous stem cells. Therefore, we also examine the current and potential clinical applications enabled by the culture of limbal stem cells.

11.2 Stem Cells of the Corneoscleral Limbus

The ocular limbus is located at the boundary between the cornea and the sclera (Fig. 11.1a, b). Anatomically, the limbus is defined by the transition from the regular collagen lamellae of the transparent corneal stroma to the opaque, irregular

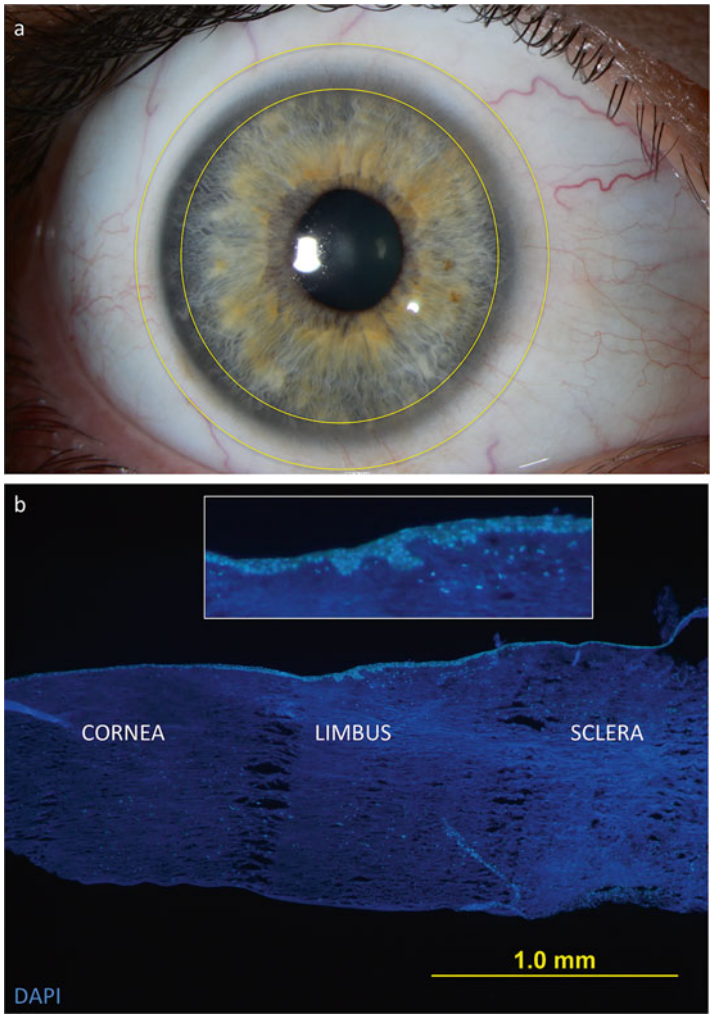

Fig. 11.1 The corneoscleral limbus. (**a**) Photograph of the human eye with the limbal region outlined in *yellow*. (**b**) Micrograph showing a frozen section of a donor human corneal rim, with cell nuclei stained with DAPI (*blue*). *Inset* shows an enlarged view of the limbal palisades. (**c**) Schematic diagram showing the cellular composition of the limbal niche. *BV* blood vessel, *LESC* limbal epithelial stem cell, *TAC* transit-amplifying cell. (**d**) Micrographs showing limbal palisades (*left panels*, *bottom right panel*) and limbal crypt structures (*top right panel*) immunostained with a pan-cytokeratin antibody (*red* signal). Cell nuclei are stained with DAPI (*blue* signal)

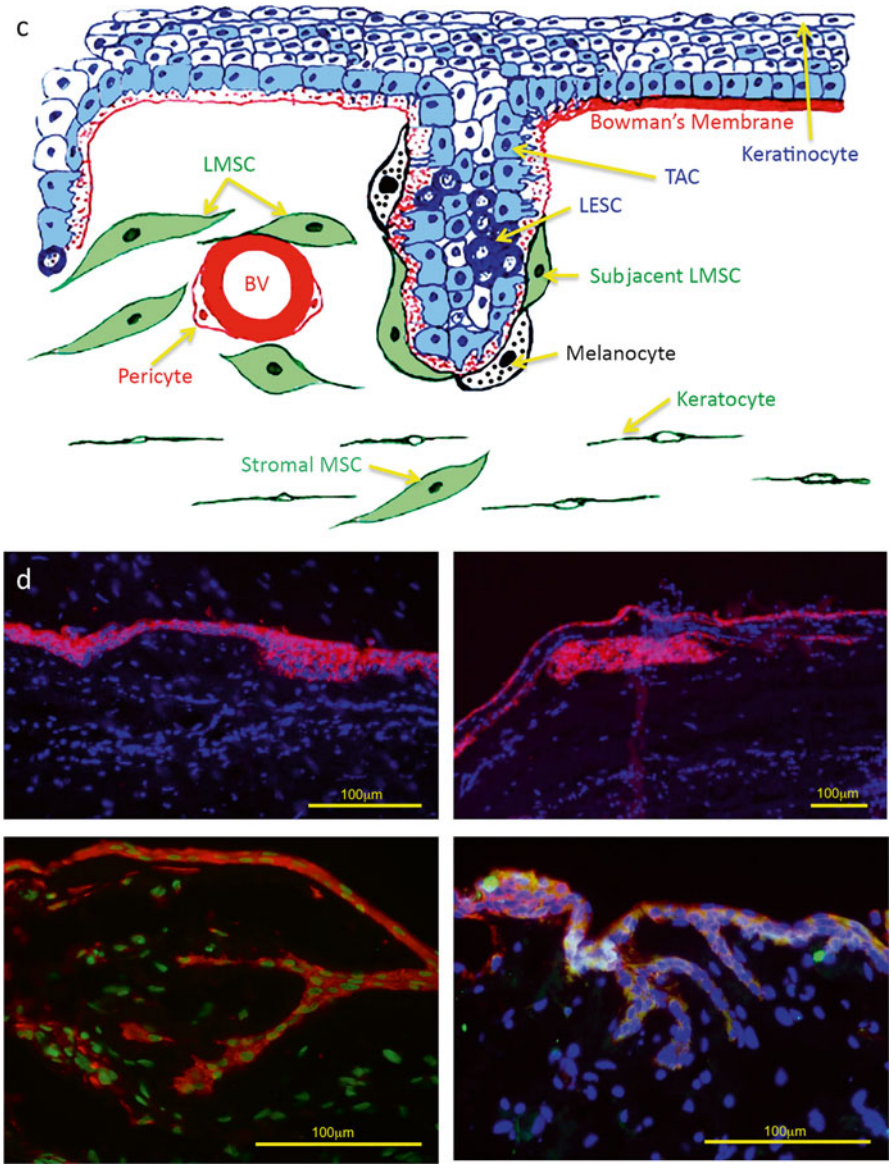

Fig. 11.1 (continued)

arrangement of lamellae in the scleral stroma. Together, the cornea and sclera form the outer coat of the eye and function to maintain the shape of the globe and protect the ocular tissues inside. The cornea is specialized for the transmission of light to the lens and retina and consists of an avascular, highly organized collagenous stroma covered by a stratified squamous epithelium. In contrast, the opaque sclera provides

strength and flexibility to the eye, contains more irregular collagen lamellae, and is covered by the loosely organized conjunctival epithelium [11].

The limbus is home to several different resident cell types. The limbal epithelium is continuous with the corneal, but not the conjunctival epithelium. The corneal and limbal epithelia consist of 5–6 cell layers of stratified columnar epithelial cells and share a common developmental origin, the optic ectoderm. In contrast, the conjunctival epithelium is derived from the extraocular ectoderm that forms the eyelids, and contains goblet cells, which secrete mucin and contribute to the tear film [12].

The limbal epithelium lies on top of the limbal stroma, which is populated with stromal keratocytes. Limbal and corneal keratocytes are derived from the craniofacial neural crest and form cell layers that produce the stromal collagen lamellae. Individual keratocytes have a dendritic morphology and form connections with neighboring cells. In contrast with the rapid turnover of limbal and corneal epithelial cells, keratocytes are a largely quiescent cell population, adopting a terminally differentiated phenotype involved in maintaining the stromal matrix [11].

In addition to epithelial cells and stromal keratocytes, the limbus contains blood vessels, nerve fibers, and melanocytes, making for a complex interface of different cell types that may contribute to the limbal microenvironment. The rich array of growth factors and matrix proteins provided by these cells form a permissive environment in which limbal stem cell populations are maintained (Fig. 11.1c).

11.2.1 Limbal Epithelial Stem Cells

The corneal epithelium is completely turned over every 7 days [13], a process that has been described by the "XYZ" hypothesis, where X represents the proliferation of basal epithelial cells, Y is the centripetal movement of epithelial cells from the limbus, and Z is the cell loss resulting from death and desquamation from the corneal surface [14]. In most stratified epithelia, proliferation occurs in the undifferentiated basal layer and differentiation of mature keratinocytes occurs as cells are displaced centripetally and vertically before being shed from the surface. In accordance with this general principle, the basal layer of the corneal epithelium consists of undifferentiated columnar cells that express the ocular transcription factor PAX6. As they migrate towards the surface, corneal epithelial progenitors differentiate into mature keratinocytes, increasing in size, downregulating PAX6 expression, upregulating the expression of cytokeratins, and forming tight junctions at their apical surfaces.

In response to minor abrasions to the ocular surface, corneal epithelial cells surrounding the debrided area are induced to proliferate and migrate, rapidly covering the exposed stroma. This process begins within minutes after the injury with epithelial cells migrating at 60–80 μm/h to seal the wound within a few hours, depending on the size of the abrasion. This early phase of corneal wound healing is mediated largely by cell migration and is followed by the induction of corneal epithelial progenitor proliferation around 24 h after the injury [15].

Table 11.1 Molecular markers in limbal cells

	LESC	TAC	Keratinocyte	LMSC	Keratocyte
ABCG2	+	−	−	+	−
P63α	++	+	−	−	−
P63	+	+	+/−	−	−
α-enolase	+	+	−	−	−
Cytokeratin-15	+	+	−	−	−
Vimentin	+	+	+/−	+	+
Nestin	−[a]	−[a]	−[a]	+	−
CD34	−	−	−	+[b]	−
βIII-Tubulin	−[a]	−[a]	−[a]	+	+
Cytokeratin-3/12	−[a]	+/−	++	−	−

[a]Expression not observed in vivo, but may be induced in vitro

[b]Expression in a subpopulation of LMSC

The corneal epithelium differs from other stratified epithelia in that it does not contain a resident stem cell population. While most stratified epithelia contain slow-cycling stem cells in their basal layer, the basal layer of the corneal epithelium contains only progenitor cells with limited proliferation potential and capacity for self-renewal in culture. These progenitors express mature keratinocyte markers, such as cytokeratins K3 and K12, and lack of expression of stem cell markers such as p63α, α-enolase, and ABCG2 (Table 11.1) [16–20].

In 1944, Ida Mann reported the movement of pigmented limbal cells towards the injury site in during corneal regeneration in the rabbit, providing the first evidence for the role of the limbus in the maintenance of the corneal epithelium [21]. Later, the limbal epithelial stem cell (LESC) niche was proposed by Davanger and Evensen, based on their observations of centripetal migration of epithelial cells from the limbus to the central cornea during epithelial wound healing [22]. Stem cells in the rodent limbal epithelium were initially identified on the basis of their BrdU (bromodeoxyuridine) label retaining properties, which identified a rare population of slow cycling cells in the basal layers [16, 22–24]. BrdU is incorporated into DNA during cell division and is commonly used to label proliferating cells [25]. In pulse-chase experiments, BrdU labeled cells were observed in the basal layers of the limbus up to 1 month after BrdU administration. Since continued cell proliferation dilutes the BrdU signal by half with each subsequent division, retention of BrdU at this time point indicates a long cell cycle in the labeled cells.

The quiescent phenotype of LESCs reduces their metabolic load, affording protection from free radicals generated by metabolic processes. Similarly, a long cell cycle protects LESCs against mutations arising from DNA replication, preserving the genomic integrity of stem cell pools by limiting the number of divisions the stem cell undergoes throughout the life of the organism. Stem cells divide asymmetrically to produce "transit amplifying cells" (TACs), which then proliferate rapidly to supply the large numbers of differentiated cells required for tissue maintenance [26].

At the limbus, the epithelium forms invaginations into the limbal stroma, known as the palisades of Vogt (Fig. 11.1b–d) [27]. Limbal palisades bring the epithelium

into proximity with underlying blood vessels, increasing the availability of vascular growth factors, and forming a specialized stem cell niche. The undulations of the limbal palisades increase the surface area of the epithelium to accommodate sufficient numbers of stem cells for corneal maintenance, as well as providing resistance to shearing forces. The limbus also contains melanocytes that protect the stem cell niche from light damage [28]. Although present throughout the limbal epithelium, LESCs are not uniformly distributed. The superior and inferior limbal regions are enriched with LESCs, locations that may further protect the stem cell pool from injury through Bell's phenomenon, a defensive reflex that causes elevation of the globes when blinking or in response to threat [16, 29]. These adaptations provide a unique and complex microenvironment that protects and nurtures the LESCs.

In addition to the palisades of Vogt (Fig. 11.1b–d), the human limbus contains specialized ingrowths of corneal epithelium extending up to 200 μm into the limbal stroma, termed limbal epithelial crypts (Fig. 11.1d, upper right and lower panels). Human limbal epithelial crypts were first identified by Dua in 2005 [30] and were found to be present at a frequency of approximately 9 per eye [31]. These specialized anatomical structures have been proposed to form an additional niche [32] and may play a role in increasing the size of the LESC reservoir.

The transition from corneal to limbal epithelium is accompanied by a number of distinct morphological and molecular changes. In the basal layer of the central corneal epithelium, which is devoid of LESCs, progenitor cells form a tight interface with the underlying basement membrane known as Bowmans Membrane (BM). The interaction between basal epithelial progenitors and BM becomes progressively altered along the central-peripheral axis of the cornea. In the central cornea, the basal surface of these progenitors is unfenestrated and smooth, while in more peripheral regions progenitors display basal surface processes that interdigitate with BM. At the limbus, these morphological changes become more distinct with limbal basal epithelial cells extending processes through the basement membrane to connect with the underlying stromal matrix [17].

The expression of extracellular matrix proteins is also altered in the limbus, with increased deposition of tenascin-C; laminin α1, α2, β1, and γ3 chains; and BM40/SPARC compared with corneal epithelium [33]. In contrast with the central cornea, the basement membrane in the limbus is less densely packed with collagen, which may aid in the diffusion of growth factors and provide a permissive substrate for the invasion of LESC fenestrations into the limbal stroma [34, 35].

A common, if not ubiquitous theme in stem cell biology is the production of TACs from rare populations of slow-cycling stem cells. In keeping with this theme, only a small proportion of limbal basal cells fulfill the criteria for a stem cell [36–38]. In most tissues, the identification of molecular markers for bone fide stem cells has proved a challenging problem due to the overlapping gene expression profiles of these cells and the early TACs they produce. TACs retain the expression of developmental genes and typically downregulate them only upon terminal differentiation. Thus, although a number of markers are known to discriminate progenitors from keratinocytes in corneal and limbal epithelia, developmental and differentiated cell makers, such as PAX6 and cytokeratins [16], have limited value in the identification of the LESCs (Table 11.1).

A number of proteins with enriched expression in the basal limbal epithelium have been proposed as specific markers for LESCs [16]. Most of these markers, including α-enolase, integrin-α9, vimentin, and nestin, are unable to discriminate TACs from LESCs in the basal epithelium. The transcription factor p63 has been shown to be essential for the proliferative competence of stratified epithelia [39] and its truncated isoform ΔNp63/p63α has been widely used to identify LESCs. TACs may retain lower levels of p63α expression [40] and a $p63\alpha^{bright}$ immunolabeling phenotype has been proposed as a selective marker of LESCs [16]. Supporting this notion, the presence of $p63\alpha^{bright}$ cells in cultured limbal epithelia was shown to correlate with corneal surface restoration after transplantation [41].

In contrast with most tissues, the hierarchy of stem cell differentiation in the hematopoietic system has been well defined and molecular markers that discriminate stem cells from progenitor cells have been identified [42]. Like stem cells in bone marrow and other tissues [43], LESCs express the universal stem cell marker ABCG2 [38, 44], an ATP binding cassette transporter that removes toxins from the cytoplasm. ABCG2 expression identifies stem cells in hematopoietic, neural, and mesenchymal tissues and is expressed in a population of slow cycling basal cells in the limbal but not corneal epithelium [37]. Since vital dyes such as Hoechst stains are actively pumped out of stem cells via ABCG2, stem cells in cell suspensions from limbal and other tissues can be detected as a 'side population' of unlabeled cells by flow cytometry [43]. Flow sorting of this side population was shown to enrich for cells with increased growth potential in culture [45]. However, since stem cell niches are typically located at the interface between different tissues, stem cells from multiple lineages may be present and the identification of stem cells using immunostaining for common stem cell markers alone could lead to the examination of a heterogenous cell population. Therefore, it is important to consider the fact that stem cells from other lineages are also present in the limbal stem cell niche.

11.2.2 Limbal Mesenchymal Stem Cells

Although ABCG2 expression is absent in the corneal epithelium, immunolabeling of human corneal sections revealed rare cells in the corneal stroma that express this stem cell marker. $ABCG2^+$ stromal cells are distributed throughout the cornea, with lower frequencies in central regions than in the peripheral cornea [46]. In the limbus, ABCG2 expression is increased in both the epithelium and the stroma, and clusters of $ABCG2^+$ stromal cells are found subadjacent to the $ABCG2^+$ basal epithelial stem cells in the palisades. These cells have been classified as mesenchymal stem cells (MSC), a population of multipotent stem cells found in almost all adult somatic tissues [47].

In contrast with the densely packed, constantly regenerating keratinocytes of the epithelium, the corneal and limbal stroma is sparsely populated with relatively quiescent keratocytes (Fig. 11.1b–d). Keratocytes occupy planes between collagen

lamellae, extending processes to connect with neighboring cells and secreting the extracellular matrix proteins that make up the bulk of the stroma. In response to injuries, keratocytes become reactive, hypertrophic, and rapidly proliferate in a scarring response that seals the injury site quickly, but at the expense of tissue organization and transparency. Corneal scar tissue is persistent and can cause long-term vision impairment if left untreated.

The human embryonic corneal stroma is populated with migrating neural crest cells during the seventh week of gestation. Neural crest cells are derived from the neuroectoderm and emerge from all regions of the neural tube to form a diverse range of mesenchymal cell types. MSC have been identified in almost all adult tissues [48] and are defined by expression of a number of cell surface markers, including CD73, CD90, and CD105, as well as the ability to differentiate into fat, cartilage and bone tissues [49]. Recently, cultured limbal stromal MSC (LMSC) were shown to conform to these criteria [47, 50]. The plasticity and growth potential of these cells, coupled with their relative accessibility and immunomodulatory functions have led to growing attention on MSC as a potential donor cell source for autologous cell therapy applications [51].

Both human and rabbit LMSC suppress lymphocyte proliferation [52], demonstrating the immunomodulatory properties of these cells. LMSC have also been shown to inhibit corneal epithelial differentiation [52], a property that may reflect their crucial role in maintaining the limbal stem cell niche.

In vivo, LMSC are present in clusters subjacent to the limbal epithelial basal layer (Fig. 11.1b–d) and are scattered at low frequencies throughout the limbal and corneal stroma. LMSC express a number of markers, including the neural lineage markers including Nestin and N-cadherin and stem cell markers such as ABCG2 [16]. MSC are thought to arise from perivascular pericytes and limbal stromal MSC have been shown to express angiogenesis markers, including CD34, CD31, α-SMA, Flk-1, VWF, and PDGFRb [50, 53]. The stem cell properties of pericytes have been reviewed elsewhere [54–56]; however, the contribution of these cells to the maintenance of the limbal niche remains largely unexplored.

11.3 Limbal Cell Culture

Since the first demonstration of limbal epithelial transplantation [57], intense focus has been directed at the development of methods for culturing limbal tissues for clinical use. A number of effective approaches to limbal epithelial cell culture for transplantation have emerged in the last decade [10, 58–60]. Although culture methods vary significantly in terms of media formulations and culture substrates, they can be broadly grouped into two main approaches: explant culture and dissociated limbal cell suspension culture.

11.3.1 Limbal Cell Culture Media

Common choices for primary limbal cell culture include standard media formulations including Dulbecco's modified Eagle's media (DMEM), MEM, and M199 serum-free keratinocyte media formulations (KSFM). Low calcium media formulations, such as KSFM, M199, MEM, have been suggested to better preserve LESCs in culture while high calcium media (DMEM, DMEM/F12) promote differentiation [10, 58, 61] (Fig. 11.2a).

In addition to basic media, primary limbal cultures require growth factor support to thrive, often supplied in the form of fetal calf serum, a potential source of xenogenic pathogens. Replacement of bovine serum with autologous human serum collected from the patient has been reported [62, 63] and defined; xeno-free, serum free media formulations such as KSFM have been developed for human epithelial cell culture [61].

11.3.2 Culture Substrate

A number of different substrates have been shown to support limbal epithelial cell growth, including human amniotic membrane [59], fibrin [58], and lens capsule [64]. Proprietary human extracellular matrix formulations, such as the Cellstart matrix (Life Technologies) also support epithelial cell growth and the maintenance of stem cell populations (Fig. 11.2a). These substrates offer xeno-free culture conditions, limiting the exposure of cultured limbal cells to products of animal origin and eliminating the risk from xenogenic pathogens.

Another popular choice for limbal cell culture substrates is the use of feeder cell layers. Growth arrested feeder cell layers provide a rich array of attachment and growth factors and have been widely used in stem cell and primary cultures. For the culture of limbal cells, the 3T3 mouse embryonic fibroblast cell line has been commonly employed and has been shown to promote enrichment of stem cells and epithelial stratification.

More recently, cultured LMSC have been shown to support limbal epithelial growth in culture [52]. Since these cells can be expanded from the same biopsies used to establish primary autologous limbal cell cultures and are involved in the maintenance of LESCs in vivo, LMSC may prove the most logical choice for feeder cell substrates in clinical limbal epithelial culture.

11.3.3 Limbal Explant Culture

In the explant culture approach, superficial limbal tissue is removed by physical dissection, preserving the architecture of the limbal cell niche. Explants are plated with the epithelial surface in contact with the culture dish to encourage epithelial

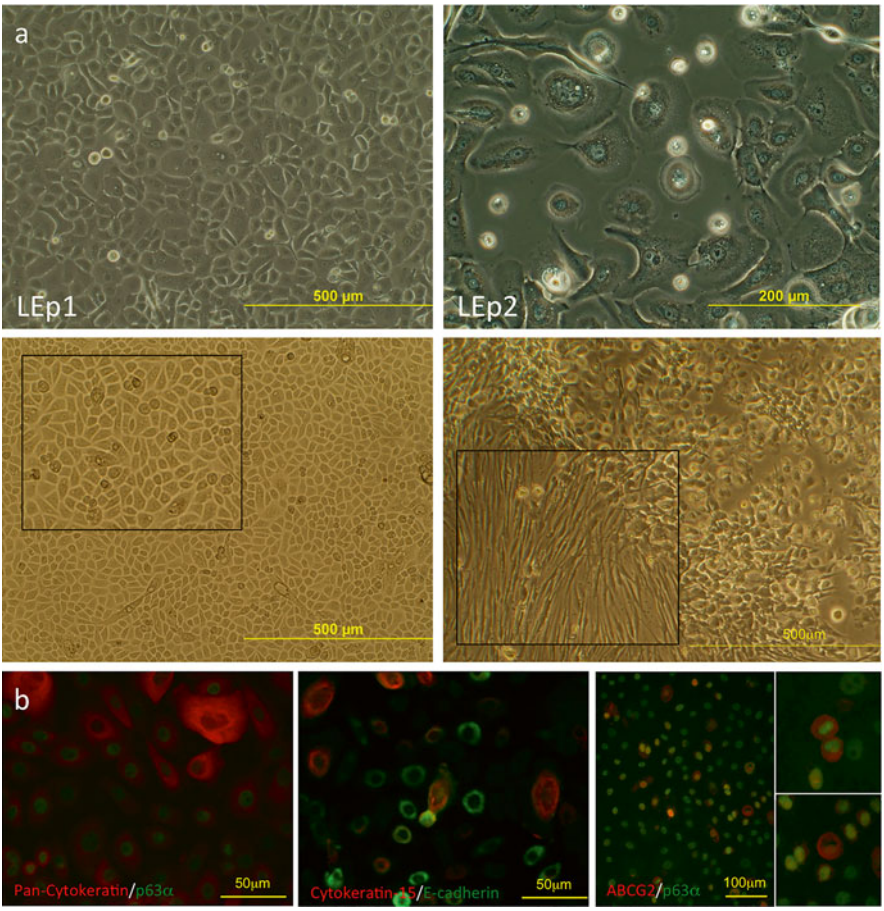

Fig. 11.2 Human limbal cell culture. (**a**) Surplus human limbal rims were obtained for culture after corneal transplantation procedures (typically 2–4 weeks postmortem). The limbal epithelium was removed after pretreatment of the entire limbal rim with collagenase for 4–6 h, a procedure known to liberate both LESCs and LMSCs. Cells were dissociated using calcium chelation and plated onto human extracellular matrix coated culture dishes in low calcium, serum free keratinocyte media (KFSM) containing EGF. Cells were passaged once per week and maintained growth and differentiation characteristics over multiple passages (*upper panels*). In high calcium media, limbal epithelial (LE) cultures differentiated into cobblestoned monolayers (*lower left panel*). Contamination of cell suspensions with stromal tissue led to mixed cultures containing rapidly proliferating fibroblast like cells (*lower right panel*). (**b**, **c**) Immunostaining of cultured limbal epithelial cells. (**b**) Fluorescence micrographs show double immunostaining of limbal epithelial cell cultures with antibodies for Pan-Cytokeratin/p63α (*left panel*), Cytokeratin-15/E-cadherin (*middle panel*), and ABCG2/p63α (*right panel*). *Insets* in the *right panel* show enlarged views of ABCG2^{+}/p63α^{bright} LESCs and ABCG2^{+}/p63α^{-} LMSCs. (**c**) Human keratinocytes cultured in KFSM exhibit immunoreactivity for neural lineage proteins, including βIII-Tubulin, Vimentin, and Nestin. (**d**) Micrographs show goblet cell (*arrow*) contamination of limbal epithelial cultures. (**e**, **f**) Human limbal cell suspensions were cultured on uncoated tissue culture plastic in high calcium, serum free neural stem cell media containing EGF and FGF2. (**e**) Micrograph shows an adherent mesenchymal stem cell colony. (**f**) Micrographs showing floating neurospheres formed in neural stem cell media

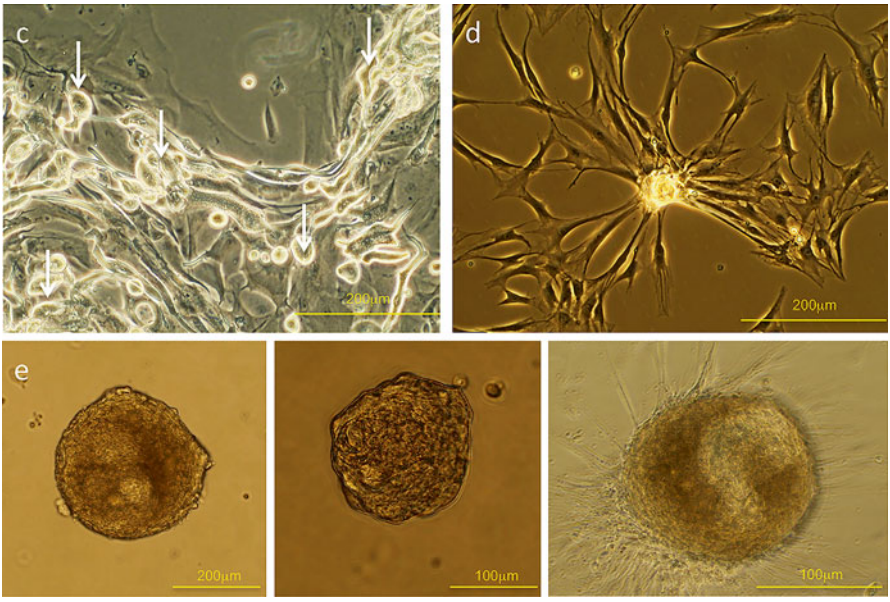

Fig. 11.2 (continued)

outgrowth; however, outgrowth may occur from resident keratocytes and limbal MSCs present in the explant [10]. The presence of these cells in explant cultures likely promotes the maintenance of LESCs, acting as endogenous "feeder cells" in the culture system.

To encourage epithelial stratification, some groups perform airlifting, a procedure in which the culture media level is lowered to the surface of the explant [58]. Under these conditions, limbal epithelial monolayers differentiate to form a multilayered epithelium.

11.3.4 Dissociated Limbal Epithelial Cultures

An alternative approach to the culture of limbal epithelium involves enzymatic dissociation of the limbal niche and culture of single cells on a suitable substrate, such as human amniotic membrane or 3T3 feeder cells. The basal cells of the corneal epithelium are anchored to a dense basement membrane while superficial keratinocytes bind to each other with tight junctions formed by calcium-dependent adhesion molecules, such as E-cadherin. For the preparation of primary limbal epithelial cell suspensions, many laboratories isolate the initial limbal tissue sample by physical dissection, followed by collagenase treatment and dissociation using trypsin or calcium chelating agents. However, the limbal epithelium is

well anchored to the underlying stroma, making its removal by physical dissection alone a technically challenging procedure. In addition to the stromal and epithelial cells that make up the limbal niche, other cell types, including melanocytes, vascular endothelial cells, as well as nerve fibers and their support cells are present in the limbal niche. Poor separation of the limbal epithelium from the stroma may result in contamination of primary cultures with stromal keratocytes and MSC. Mixed limbal epithelial/stromal cultures form disorganized cellular monolayers, with patches of squamous epithelial cells surrounded by rapidly proliferating, fibroblast-like cells (Fig. 11.2a).

Preincubation of entire limbal rims with enzymes such as dispase or collagenase prior to dissection facilitates the removal of the limbal epithelium (Fig. 11.2a–c). In our laboratory, we isolate the limbal epithelium by incubating corneoscleral rims in collagenase for 2–4 h. This treatment allows the removal of epithelial sheets for subsequent dissociation; however, care must be taken to ensure that any residual conjunctival epithelium present on the donor rim is not collected. The presence of goblet cells in limbal epithelial cultures indicates the contamination with conjunctival epithelium (Fig. 11.2d).

Dissociated limbal epithelial cells require a suitable substrate for attachment and growth. Different culture substrates that support the formation of epithelial monolayers include human amniotic membrane, 3T3 feeder cells, and silk fibroin [1, 7, 10, 65, 66]. Clonal culture of limbal and corneal epithelial cell suspensions on feeder cell layers demonstrated the presence of three classes of proliferative cells, each producing colonies of different sizes. Meroclones and paraclones have limited proliferative and self-renewal potential, producing small colonies after several weeks in culture. In contrast, holoclones generate large colonies and can be serially passaged to produce large numbers of cells, a property attributed to the presence of LESCs. Limbal epithelial cell suspensions give rise to mero-, para-, and holoclones, while corneal epithelial cell suspensions generate only mero- and paraclones [36].

In our laboratory, we have used a commercially available serum and xeno-free free, low calcium keratinocyte media and extracellular matrix kit that supported limbal cell growth over multiple passages without changes in epithelial phenotype. Switching to a high calcium media promotes differentiation of epithelial monolayers, with tight cell–cell junctions and a cobblestoned appearance (Fig. 11.2a). Cultured keratinocytes express typical corneal epithelial markers, including increased cytokeratin and E-cadherin staining in larger cells compared with smaller progenitor cells expressing cytokeratin-15 (Fig. 11.2b).

The presence of small, rounded cells expressing ABCG2 and $p63\alpha^{bright}$ in these cultures is consistent with the maintenance of LESCs in this culture system (Fig. 11.2b, right panel). In addition to $ABCG2^{+}/p63\alpha^{bright}$ cells, we observed $ABCG2^{+}/p63\alpha^{-}$ cells in limbal epithelial cultures, which likely reflect the presence of LMSC in these cultures. Li et al. examined the effect of limbal epithelial isolation using collagenase or dispase and showed that both enzymes are able to separate the epithelium from the stroma; however, different types of cells were isolated by each enzyme [50].

11.3.5 Culture of Limbal Mesenchymal Stem Cells

With many early studies focused on LESCs, the presence of MSCs in the limbal stroma and their potential contribution to primary limbal cultures was largely unappreciated until recently [7, 50]. The close apposition of LESCs and LMSCs in the limbal niche makes the isolation of one population a challenging proposition since removal of the limbal epithelium by physical scraping likely results in the collection of subjacent LMSC as well as basal LESCs.

Investigations performed by the laboratory of Tseng have shown that different enzymes treatments isolate different populations of human limbal niche cells. Dispase treatment was shown to isolate sheets of limbal epithelium [67], while collagenase treatment was shown to isolate epithelial cells as well as LMSCs and stromal cells in the limbal niche [68]. Removal of the limbal epithelium using dispase, followed by collagenase treatment of the exposed stroma was shown to enrich for LMSC, which formed floating cell clusters during digestion, and pericytes, which were adherent to the tissue culture plastic [50]. Together, these studies provide a useful set of methods for the isolation and culture of different limbal stem cell populations.

LMSC can be cultured under conventional MSC culture conditions in which adherent cells are grown on tissue culture plastic in DMEM media supplemented with FCS. Under these conditions, LMSC tend to lose the expression of stem cell markers after several passages, suggesting that standard MSC cell culture conditions may not be optimal for the maintenance of LMSC. In contrast, a low calcium, M199 based media (with FCS supplementation) was shown to preserve stem cell marker expression in cultured LMSC [69]. In our laboratory, we employ a DMEM/F12-based, serum free neural stem cell media containing EGF and FGF2 for culturing LMSC (Fig. 11.2e).

LMSC can be expanded over many passages and can be differentiated into osteoblasts, adipose cells, and chrondrocytes [47, 50]. LMSC express EGF- and FGF-receptors as well as neural lineage proteins such as Nestin, vimentin, and βIII-tubulin. Like neural crest-derived stem cells found in other tissues throughout the body [70–72], LMSC form neurospheres in the presence of FGF2 and EGF (Fig. 11.2f) and can be differentiated into cells displaying the characteristics of functional neurons [50, 60, 73].

11.4 Plasticity of Limbal Stem Cells

In vivo, LESCs perform as unipotent, rather than multipotent stem cells and the differentiation of their progeny is limited to the corneal keratinocyte phenotype. Cultured LESCs are able to generate stratified corneal epithelium and the use of appropriate culture conditions can preserve this capacity over many passages, without alterations in the phenotype of differentiated cells. Although a number of claims regarding the transdifferentiation potential of limbal stem cells have been made in the past decade, including neural lineage induction [74] and pluripotency [7], many of these studies employed culture methods that isolate both LESCs and LMSCs.

The presence of multiple stem cell lineages in the limbal niche and in primary limbal cell cultures has complicated the interpretation of data obtained from primary limbal cell cultures, particularly with regard to differentiation potential. Overall, direct evidence for LESC multipotency is lacking, while evidence for the plasticity of LMSC is gaining strength.

11.4.1 Neural Lineage Induction

During neurulation in the human embryo, inhibition of bone morphogenic protein (BMP) signaling in ectodermal progenitors causes induction of these cells into the neural lineage and the formation of the neuroectoderm. This process has been recapitulated in vitro to induce neurulation in embryonic stem cell cultures using the bone morphogenic protein (BMP) receptor antagonist Noggin [75].

In 2002, Zhao et al. reported the formation of floating neurospheres from rat limbal epithelial suspensions cultured in neural stem cell media containing EGF, FGF2, and Noggin [4]. In this report, debrided limbal stromal tissue failed to produce neurospheres, leading the authors to conclude that LESCs were the source of limbal neurospheres (LiNS). Since BMPs were found to regulate the expression of the neural progenitor marker Nestin in cultured limbal neurospheres, it was proposed that LESCs retained a primitive phenotype resembling the primitive ectodermal progenitor cells they are derived from and, like embryonic stem cells, could be induced into the neural lineage by inhibition of BMP signaling [4].

Although developmental recapitulation is an attractive hypothesis, the presence of LMSC in the limbal niche was largely overlooked in these studies. The physical dissection methods used by Zhao et al. to isolate the limbal epithelium lead to the isolation of LMSC as well as LESC (see above). Furthermore, physical debridement of the limbal epithelium likely removes subjacent LMSC as well, leaving only keratocytes and rare MSC in the exposed stroma. Other laboratories have demonstrated neurosphere induction in primary limbal suspensions using EGF and FGF2 alone. Both epithelial and stromal cell suspensions formed LiNS in the absence of Noggin, although stromal cells gave rise to neurospheres at lower frequencies [50]. These results suggest that the neural crest derived limbal MSC, which are enriched at the limbal palisades, but are also scattered at low frequencies throughout the corneal and limbal stroma, are the neurosphere forming stem cells present in the limbal niche.

The colocalization of the epithelial marker p63 with the neural progenitor marker Nestin and neurotransmitter receptors for GABA, glycine, and serotonin in human limbal explant cultures was described in 2003 [5, 74], lending further support to the suggestion that LESCs possess competence for neural lineage induction. However, expression of neural markers such as Nestin, Vimentin, and βIII-tubulin may be induced in keratinocyte progenitors in culture without apparent loss of keratinocyte phenotype (Fig. 11.2c). Furthermore, neurotransmitter receptors are expressed in nonneural cells found in other tissues, including the corneal epithelium [76] and electrophysiological readings failed to detect action potentials in putative neuronal cells [4, 74].

While direct evidence for Noggin-dependent neurogenesis from LESCs is lacking, the neural potential of LMSC is becoming increasingly established. LMSC are derived from neural crest cells, which are derived, in turn, from the neuroectoderm. Thus, LMSC are derived from cells that underwent developmental specification into the neural lineage. Culture of primary MSC from bone marrow, adipose, skin [70–72], in the presence of EGF and FGF2 has been shown to produce neurospheres, suggesting neurosphere formation is a universal property of MSC. Given these observations, the establishment of LiNS cultures by LMSC can be seen as part of their neural crest heritage, rather than transdifferentiation, which is defined as the re-specification of cellular phenotype across different developmental lineages.

MSC are partly defined by their ability to undergo osteogenesis, adipogenesis, and chondrogenesis [49], and LMSC have been shown to possess this plasticity, producing bone, fat, and cartilage in vitro [47, 53]. The plasticity of MSC is further indicated by their competence for neural lineage induction and LiNS have been shown to differentiate into cells expressing markers for mature neurons (MAP2, neurofilament), astrocytes (glial-fibrillary-acidic protein), and oligodendrocytes (04) [4] in vitro. Coculture of LiNS with embryonic hippocampal cells induced neuronal differentiation, while coculture with neonatal retinal cells induced photoreceptor differentiation [5]. Transplantation of LiNS into the rat retina in site directed differentiation of donor cells into photoreceptors [6]. These properties are consistent with the phenotype of neural stem cells derived from other tissues, making LiNS an attractive target for therapeutic applications.

11.4.2 Induction of Pluripotency

Pluripotency refers to the capacity for differentiation into all the developmental lineages of the embryo, excluding the extra-embryonic tissues. In vivo, pluripotent stem cells are found in the inner cell mass of the early embryo, and the development of pluripotent stem cell culture techniques has led to the establishment of human embryonic stem (hES) cell lines as well as the publication of numerous protocols for directed differentiation of these cells into many types of somatic cells [77]. Although hES cell-based regenerative therapies are being developed for the treatment of ocular diseases [78], the allogenic nature of these cells may limit the efficacy of this approach and long-term immunosuppression may be required to prevent graft rejection.

In 2006, the development of methods for inducing pluripotency in cultured adult fibroblasts by retroviral expression of the transcription factors Oct4, Sox2, c-myc, and Klf4 [79] provided the foundation for the new field of cellular reprogramming [80, 81]. Induced pluripotent stem (iPS) cells have been shown to possess a phenotype similar to embryonic stem cells, displaying self-renewal and differentiation into all somatic cell types. Reprogramming of adult somatic cells to pluripotency has great potential for regenerative medicine, allowing the establishment of patient-matched pluripotent stem cell lines for autologous cell therapies [82].

Since 2006, a variety of methods for inducing transcription factor expression in adult primary cultures have been developed that may be suitable for clinical implementation, including transfection of episomal plasmids, mRNA, micro-RNA, and the delivery of recombinant proteins [83–87]. These approaches reduce the risks of mutation by avoiding permanent genetic modification of the donor cells. However, forced expression of transcription factors, including oncogenes such as c-myc and KLF4 may lead to altered or even transformed cellular phenotypes. The possibility of incomplete reprogramming, evidenced by the retention of donor cell gene expression profiles and activation of genes involved in cellular immunity in putative iPS cells [88], necessitates the use of stringent quality control during the production of iPS cell lines, greatly increasing the time and cost involved.

Since stem cells from various origins may already express some of the additional factors associated with the pluripotent state, such as Sox2, KLF4, c-myc, and Nanog, the selection of suitable donor cells for reprogramming has been shown to reduce the number of factors needed for the induction of pluripotency [89, 90]. In the search for appropriate donor cell sources for reprogramming, the ocular limbus has been drawn into the spotlight with recent reports of dedifferentiation of limbal cells into a pluripotent state using cell culture methods alone [7, 9].

In 2005, Dravida et al. reported the expression of pluripotency gene *OCT4* in the stromal compartment of human limbal explants cultured in matrigel in the presence of FGF4 and LIF. Using this culture system, these authors isolated 'limbal fibroblast-like cells' that expressed the stem cell antigen SSEA4 and differentiated into cells of neural, pancreatic, adipose, osteoblast, cardiomyocyte, chondrocyte, and hepatocyte lineages [8].

Later, Balasubramanian et al. reported the induction of pluripotency in rodent LiNS cultures without the delivery of exogenous transcription factors. Culture of LiNS in mouse embryonic stem (mES) cell-conditioned media resulted in the formation of colonies of cells expressing Oct4 and Nanog. Like mES cells, rat limbal iPS cells (LiPSCs) formed teratomas when injected into immunodeficient mice and could be differentiated into neurons, cardiomyocytes, and hepatocytes using directed differentiation protocols developed for hES cells [7]. Later reports from the same group showed that injection of mouse LiPSCs into blastocysts led to the generation of chimeric mice; however, germ line transmission was not demonstrated. LiPSC clones were found to undergo senescence after six passages, which may indicate a more limited expansion potential compared with embryonic stem cells [9].

The induction of pluripotency in limbal stem cells by soluble factors produced by mES cells represents a significant advance in regenerative medicine, opening the possibility of reprogramming adult stem cells to pluripotency without the need for direct genetic manipulation. The use of appropriate, accessible stem cell populations and the ability to manipulate endogenous pluripotent gene expression using culture conditions alone could provide an ideal source of autologous cells for cell therapies. However, identification of the mES cell-derived signals that induce pluripotency will be essential to produce defined protocols suitable for clinical implementation. Parameswaran identified mES-derived exosomes containing micro-RNAs (mir294, mir295, and mir302) known for enhancing or inducing pluripotency in

fibroblasts [84], providing one potential source of reprogramming signals [9]. However, further characterization and validation by other laboratories is required to understand the mechanism of LiPSC induction and to demonstrate the conservation of LiPSC induction between humans and rodents.

Building on the work of Zhao et al. [4, 5], it was concluded that the generation of LiPSC from rodent LiNS was dependent on Noggin-induced dedifferentiation of LESCs. However, as discussed earlier, the evidence for this conclusion remains open to interpretation. The LESC origin of LiNS and LiPSC was implicated by the expression of the epithelial stem cell markers p63α and α-enolase in early LiNS. However, since other laboratories have shown that both LESCs and LMSCs may be present in limbal cell isolates [9, 50, 60], and expression of LESC markers is progressively lost with LiNS and LiPSC passaging, the role of LESCs and Noggin in the induction of LiPSCs remains unclear.

Both the Oct4 and Nanog promoters have been shown to be hypomethylated in LiNS and LiPSC cultures, consistent with inducible expression [9]. Chromatin immunoprecipitation analysis further demonstrated the association of trimethylated histone H3K4 with Oct4 and Nanog promoters in LiPSC cells, a characteristic associated with active gene expression. In comparison, in LiNS, these promoters were found to contain trimethylated H3K27 histones, which is associated with transcriptional silencing. Several authors have reported the presence of Oct4 mRNA in human limbal tissues [91, 92] and other stem cell populations [93–95], although the significance of these observations has been questioned [96, 97]. Expression of Oct4 mRNA was very low in limbal cells and cultures compared with embryonic stem cells [92] and although Oct4 immunoreactivity in basal cells of the limbal epithelium was reported [91], later studies failed to find evidence of Oct4 protein in the limbus [92]. Recently, Oct4, Sox2, and Nanog immunoreactivity were demonstrated in human limbal cell suspensions and cultures [68]. Oct4 protein immunoreactivity was observed in both vimentin$^+$ cells as well as p63α$^+$ cells, suggesting both LMSCs and LESCs may express pluripotency genes. Furthermore, expression of Oct4 was enhanced by culture in mES cell conditioned media [60] suggesting that induction of pluripotency through cell culture methods alone may be conserved between humans and rodents.

Together, these results indicate that stem cells in the limbus have the capacity for the induction of key pluripotency genes, although it remains unclear whether LESCs, LMSCs, or both cell types may contribute to LiPSC induction. The use of recently developed methods for preferential culture of the various stem cell populations in the limbus [50, 60, 68] will be important for future studies aimed at answering these questions.

11.5 Clinical Applications

With its superficial location, the limbus is an accessible target for harvesting stem cells from human patients. Limbal biopsy has been shown to be a safe and simple procedure with minimal discomfort or risk to the patient [98]. Transplantation of

cultured limbal tissues for the treatment of limbal stem cell deficiencies (LSCD) has been performed since 1997 and has proved an effective treatment for this disease. However, the mechanism by which limbal transplants restore corneal transparency remains unclear. A lack of long-term donor cell survival has been reported in successfully treated patients, suggesting that the replacement of limbal stem cell pools with donor LESCs is not the underlying therapeutic principle. Alternate explanations may include the remodeling of the limbal stem cell niche under the influence of healthy limbal tissue, which could support LESC repopulation from residual host stem cell pools.

11.5.1 Limbal Epithelial Cell Transplantation

Damage to the limbal stem cell niche can occur through chemical burns, microbial infections, repetitive surgeries, adnexal abnormalities of the eyelids or lacrimal system [99], or genetic diseases such as Stevens–Johnson syndrome [2]. These types of injuries lead to LSCD, which can be total (encompassing the whole cornea) or partial (localized to a limbal region) depending on nature of the defect and the extent of the damage.

LSCD is a debilitating and painful condition resulting in the destabilization and loss of corneal epithelium and the ingrowth of the vascular conjunctival epithelium over the cornea. Encroachment of the conjunctiva reduces corneal transparency, leading to loss of vision in the affected eye. The treatment of LSCD by transplantation of donor limbal tissue into the patient was first reported in 1989; however, this technique required large sheets of limbal epithelium, the procurement of which comes with the risk of inducing LSCD in the donor eye [57]. With the development of limbal culture methods, it was later shown that small limbal biopsies could be expanded in vitro to produce sufficient quantities of epithelial cells for transplantation [100]. In a recent study, limbal biopsy was shown to be a relatively safe and simple procedure that posed little risk to the patients [101]. Thus, small amounts of limbal tissue can be harvested from an unaffected eye or limbal region from the same patient (or close relative) for expansion in culture and transplantation into the LSCD affected eye.

Since 1997, this approach to the treatment of LSCD has been widely explored, and several reviewers have recently examined the clinical efficacy of cultured limbal epithelial cell transplants [1, 3, 102]. Despite a plethora of different LSCD causes, donor cell sources, cell harvest and culture techniques, surgical methods, and postoperative management regimes, the clinical success rates of limbal transplantation is relatively high, with most reviewers reporting a 75 % success rate across different studies. Clinical failures usually presented within the first year or two after transplantation [1, 103]. In one study, the success of limbal transplantation was correlated with the number of $p63\alpha^{bright}$ LESCs present in limbal cultures, and these authors suggested that cultures with fewer than 3 % LESCs were associated with low clinical success rates [41].

11.5.2 The Therapeutic Mechanism of Limbal Cell Transplantation

Limbal transplantation was developed with the idea that replacing the depleted LESC pool with new donor cells would restore the regenerative capacity of the corneal epithelium. Although the clinical success of this technique in restoring corneal transparency supported this theory, Daya et al. demonstrated a lack of donor cell DNA on the corneal surface 9 months after limbal stem cell therapy [104]. Other authors have also described the preservation of corneal transparency in the absence of long-term graft survival [3], bringing the proposed mechanism for this cell therapy into question.

Alternate explanations for the efficacy of limbal transplants could include the initial remodeling and reconstruction of a scarred limbal niche by donor cells and/or host cells responding to the surgery, followed by the repopulation of the limbal basal epithelium with LESC. Since the corneal epithelium of transplanted patients does not contain donor cell DNA, replaced LESCs must be derived from the host. However, considering LSCD is defined by the loss of LESCs, the source of new LESC remains unclear.

Repopulation of the limbal niche could be achieved by symmetric proliferation of rare residual LESC or through dedifferentiation of keratinocyte progenitors. Recently, an additional possibility was suggested by the identification and characterization of a population of $CD34^+$ limbal stromal MSC. These cells were shown to differentiate into corneal epithelial cells under keratinocyte culture conditions [69], suggesting LMSC may be another potential source of new LESCs in vivo.

Regardless of the source of new LESC, the clinical outcome of limbal cell transplantations is likely to be dependent on the reestablishment of a permissive environment for LESC survival and growth. In fact, given the lack of donor LESC contribution to the restored corneal surface, this may be the only important outcome for clinical success. In this light, the reported correlation between LESC numbers in cultured grafts and clinical success [41] may reflect the fact that culture conditions that better preserve LESCs may also maintain LMSC populations, leading to a more complete recapitulation of the limbal microenvironment. Grafting of healthy limbal tissue may help restore lost signaling and matrix molecules to promote the regeneration of the niche. While the absence of host DNA on the corneal surface suggests the loss of grafted LESCs, it remains unclear whether transplanted stromal cells and LMSC survive for longer periods of time. The immunosuppressive properties of LMSC, together with their ability to prevent corneal epithelial differentiation [52] suggest that LMSC could play an important role in regenerating the limbal niche.

The therapeutic potential of LMSC remains largely unexplored; however, these cells may already play a previously unappreciated role in limbal cell transplantations. Together with their accessibility and growth potential, the plasticity of LMSC opens up the potential for autologous cell therapies for other diseases in the eye and beyond.

11.6 Concluding Remarks

With the identification of multiple stem cell pools in the human ocular limbus and the development of safe and effective methods for selective culturing of these cells [10, 50, 60, 68], exciting possibilities lie ahead in limbal stem cell research and regenerative medicine. A deeper understanding of the plasticity of these stem cells and the therapeutic mechanism behind limbal stem cell transplantation will inform the development of new approaches to the treatment of LSCD and other ocular disorders.

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Chapter 12
Advances on Optic Nerve Regeneration and Therapeutic Strategies

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A. Pébay (ed.), *Regenerative Biology of the Eye*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4939-0787-8_12, © Springer Science+Business Media New York 2014

Abbreviations

ATF-3	Activating transcription factor 3
BDNF	Brain-derived neurotrophic factor
Bim	Bcl-2 interacting mediator of cell death
cAMP	Cyclic adenosine monophosphate
Ch	Chiasm
CHOP	CCAAT/enhancer-binding protein-homologous protein
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CP	Cerebral peduncle
CSPG	Chondroitin sulfate proteoglycan
CTB	Cholera toxin B fragment
DLG	Dorsal lateral geniculate nucleus
dLGN	Dorsal lateral geniculate nucleus
DLK	Dual leucine zipper kinase
DRG	Dorsal root ganglion
FGF2	Fibroblast growth factor
GAP-43	Growth associated protein-43
GDNF	Glial-derived neurotrophic factor
GTPase	Guanosine triphosphate hydrolase
Jak	Janus kinase
KLF	Krüppel-like factor
KSPG	Keratin sulfate proteoglycan
LAR	Leukocyte common antigen-related phosphatase
LGN	Lateral geniculate nucleus
LINGO1	Leucine rich repeat and Ig domain containing 1
MAG	Myelin glycoprotein
MAPK	Mitogen-activated protein kinases
MTN	Medial terminal nucleus
mTOR	Mammalian target of rapamycin
NgR	Nogo receptor
Ocm	Oncomodulin
Omgp	Oligodendrocyte-myelin glycoprotein
OMR	Optomotor response

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OPT	Tract
OPT	Olivary pretectal nucleus
PI3 kinase	Phosphatidylinositide 3-kinase
PN	Peripheral nerve
PNS	Peripheral nerve system
PTEN	Phosphatase and tensin homolog
PTPσ	Transmembrane protein-tyrosine-phosphatase sigma
Puma	p53 upregulated modulator of apoptosis
RGC	Retinal ganglion cells
RhoA	Ras homolog gene family member A, a small GTPase protein
ROCK	Rho-associated protein kinase
SC	Superior colliculus
SCN	Suprachiasmatic nucleus
SOCS3	Suppressor of cytokine signaling 3
SPRR1A	Small proline-rich protein 1A
STAT	Signal transducer and activator of transcription
TROY	TNF receptor family member
vLGN	Ventral lateral geniculate nucleus
Zym	Zymosan

12.1 Introduction

The optic nerve can be affected by neurodegenerative diseases that can lead to blindness. Glaucoma is one of the neurodegenerative diseases that affects millions of people worldwide and is the prevalent cause of vision loss. A traumatic injury to the optic nerve mimics the effects of some disease in the eye. It causes degeneration of nerve fibers that connect the eye to areas inside the brain responsible for the processing of visual information. The degeneration of optic nerve fibers is an irreversible event, and many studies have tried to investigate the mechanisms that lead to failure of the regenerative capacity of those neurons. In the last decades, an immense progress has been made on the discovery of key molecules that impede or stimulate the regeneration of optic nerve fibers. Some important pathways that are involved in the event of cell death and inhibition of cell intrinsic growth capacity have been unraveled. Following these discoveries, studies have tried to combine different types of therapeutic strategies to increase the levels of regeneration and survival of the main cells that connect the eye to the brain, the retinal ganglion cells (RGCs).

12.2 Retinal Ganglion Cell Survival

Some molecules play a major role in RGC survival, and it is known that after an injury to rats' optic nerve the rate of RGC survival decreases over 60–70 % within 2 weeks, and 1 month after lesion only 5 % of RGC survive [1]. Studies performed in rats showed that there are pro-survival molecules that were reported to increase

survival of these cells after an injury. Some trophic factors, such as brain-derived neurotrophic factor (BDNF) and neurotrophin (NT) 4/5, are known to improve RGCs survival, and they partially protect these cells from death after nerve injury [2–5]. Overexpression of antiapoptotic protein Bcl-2 in mice increases survival of RGCs 1 month after optic nerve crush; on the other hand, no regeneration of optic nerve fibers was observed after this treatment [6].

Other important molecules are the ones from the caspase family, which also interfere on RGC survival after a lesion. There is an increase on caspase-3 expression 10 days postlesion and its inhibition showed to be effective in early stages after the injury, when 30–35 % of RGCs were rescued from apoptosis in rats [7]. Other members of the caspase family are also overexpressed after lesion, the caspase-6 and -8, and their inhibition is neuroprotective to RGCs, promoting a three fold increase of RGC survival after optic nerve injury compared to control animals. Further, their inhibition also stimulates regeneration of the optic nerve fiber, a phenomenon that it is not observed when inhibiting caspase-3 [8].

More recently, a new molecule was discovered in studies that investigated pathways orchestrating peripheral nerve degeneration [9]. It was found that a MAP3 kinase—dual leucine zipper kinase (DLK) is involved in axonal growth, apoptosis, and neural degeneration during development and in many neurodegenerative disorders [10]. After these discoveries, some studies on the optic nerve described its role on survival and regeneration of RGCs. Indeed, in vitro, DLK levels increase within 18 h of culture initiation, and in vivo, 2 weeks after transection there are only 12 % of surviving cells in mice [11]. Overexpression upregulated modulator of apoptosis (*Puma*) and *Bcl-2* interacting mediator of cell death (*Bim*). At the same time, there is also an upregulation of regeneration-associated genes: *ATF-3*, *Sprr1a*, *Klf6* [12]. These effects can be partially reversed when a DLK inhibitor is applied in the eye. It increases the rate of RGC survival—32.3 % 2 weeks after transection [11] and only few fibers regenerate after the lesion site. These results show that DLK role is controversial as it activates signaling pathways involved with both cell death and axonal regeneration. Its inhibition can improve one aspect and can be detrimental to the other, but both are important for functional recovery.

Some of these studies showed that improvement of RGCs survival alone can be partially effective in stimulating optic nerve regeneration, and these effects do not account for functional recovery [8, 13], once the amount of axonal regeneration is not sufficient to reconnect the eye with visual targets in the brain.

12.3 Optic Nerve Regeneration

Studies performed by Aguayo and colleagues demonstrated that CNS neurons can regenerate when put into a peripheral nervous system (PNS) environment. They showed in a series of studies with rodents that axons can grow over long distances through a peripheral nerve (PN) graft and that regenerating neurons were able to

connect to appropriate targets, synapse onto those neurons, and presented electrophysiological responses [14–18].

The outcomes from these studies implied that the PNS environment was more permissive than that found in the CNS, and this was related to the lack of inhibitory molecules that block axons elongation. However, a new hypothesis was raised by Berry and colleagues [19]. They believed that what Aguayo's group showed was mainly because of trophic factors secreted by Schwann cells. In an attempt to prove this hypothesis, they inserted a segment of PN graft into the eye of adult rats and found that this approach induced axonal regeneration in the optic nerve itself [19]. In addition to axonal regeneration, they also showed an increase in inflammatory cells in the eye in both experimental and control groups, where the latter had an acellular graft implanted (PN segments subjected to cycles of freeze and thaw to kill the cells in the graft) and the former had a cellular graft. Few years after Berry's discoveries, Leon and colleagues [20] investigated the role of intraocular inflammation on optic nerve regeneration. They showed that lens injury or an intraocular injection of a monocyte activator—zymosan—was sufficient to induce regeneration of RGCs [20]. Investigation on changes in the profile of gene expression in RGCs stimulated by inflammation demonstrated the upregulation of proregenerative genes, such as *gap-43*, sprr1, and other genes related to regeneration of dorsal root ganglion (DRG) neurons [21].

Many studies have reproduced the same effect of inflammation-induced regeneration on RGCs of mice and rats [22–25] and in 2006 our group discovered the new trophic factor Oncomodulin, which is responsible for the changes on the intrinsic growth state of RGCs that were exposed to inflammation. This effect is observed when two other molecules are present, forskolin and mannose—which are constituents of the vitreous body—the former being responsible for elevating the levels of cAMP [26, 27]. Oncomodulin promotes better axonal outgrowth than other known trophic factors, such as BDNF, ciliary neurotrophic factor (CNTF), fibroblast growth factor (FGF2), and glial-derived neurotrophic factor (GDNF). The effect of oncomodulin on cell survival, however, is not as dramatic as the one seen on axonal regeneration. There is only a twofold increase on survival of RGCs 2 weeks after optic nerve lesion [27].

After lens injury or intraocular injection of zymosan there is an increase on the levels of oncomodulin in the vitreous body, the retina [27], and in inflammatory cells [28]. After inducing inflammation both macrophages and neurophils that enter the vitreous body express oncomodulin. Although the number of neutrophils is much higher than macrophages at 12 and 24 h after zymosan injection, the amount of oncomodulin per cell is higher in macrophages than in neutrophils [28]. Using a blocking peptide for oncomodulin receptor or a neutralizing antibody antioncomodulin, the inflammation-induced regeneration of optic nerve is completely abolished (Fig. 12.1) [27]. Taken together, these results show that oncomodulin plays a central role on regeneration of RGCs stimulated by inflammation [26, 27]. However, other factors may also be involved in optic nerve regeneration after inflammation, because (1) for oncomodulin to bind to its receptor it is necessary that the levels of cAMP is increased; and (2) the rate of cell survival does not change when using a blocking

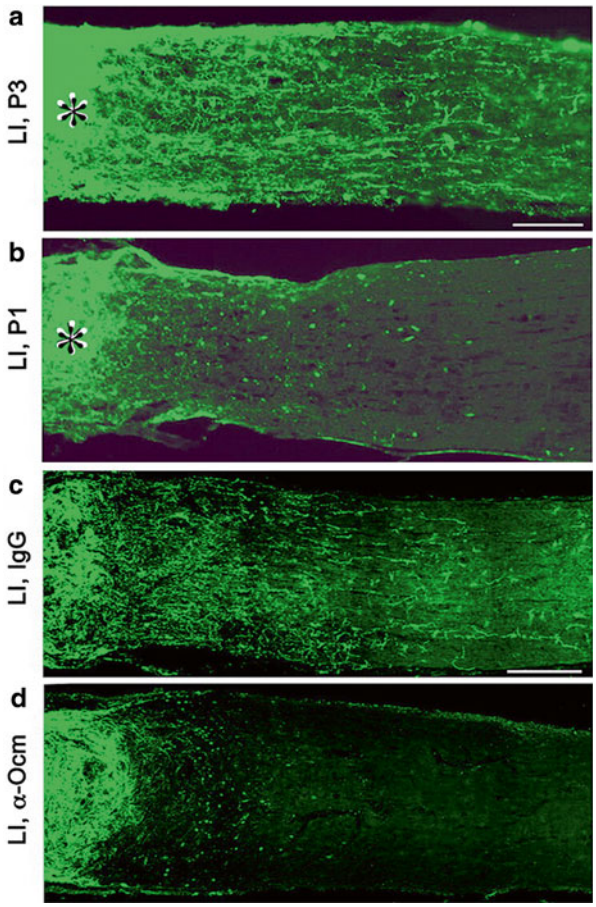

Fig. 12.1 Intraocular inflammation and Oncomodulin (Ocm) promote optic nerve regeneration. GAP-43 immunostaining shows regenerating axons in longitudinal sections through the mature rat optic nerve 2 weeks after injury. (**a**–**d**) Loss-of-function experiments. Injection of peptide P1 competes with Ocm for receptor occupancy and suppresses inflammation-induced regeneration (**b**), while control peptide P3 has no impact on axon regeneration (**a**). After optic nerve injury alone there is no regeneration, the immunostaining presents the same pattern that we see in the loss-of-function experiments. A neutralizing antibody against oncomodulin abolishes axon regeneration induced by lens injury (**d**), while a control experiment using IgG shows the expected effect of nerve regeneration induced by inflammation (**c**). Scale bar: 200 **μ**m [27]. Reproduced with permission from the Proceedings of the National Academy of Sciences of the United States of America (PNAS)

peptide for oncomodulin. These results suggest that there are other factors responsible for the elevation in cAMP levels and the increase of cell survival [29]. These unknown factors may be involved in distinct pathways, as reports on improvement of cell survival does not always show a relationship with axonal regeneration, as for

instance the experiments with overexpression of Bcl-2 improve the rate of RGC survival but do not stimulate axonal regeneration [6].

It is important to point out that there is a claim in the literature affirming that the effect of inflammation on axonal regeneration is due to the trophic factor CNTF [30, 31]. Many groups have tried to reproduce this effect but failed to do so [20, 25, 27, 32–34]. Nevertheless, CNTF has been shown to exert a chemotactic effect on blood-borne monocytes, but it cannot stimulate neurite outgrowth in retinal explants [35]. After optic nerve injury or intraocular inflammation [21] and after intraocular injection of CNTF, there is an increase in the levels of SOCS3, a suppressor of the Jak-STAT pathway, the pathway on which CNTF exerts its effect [36]. This can be one of the reasons why CNTF cannot stimulate regeneration of RGCs.

12.4 Cell Extrinsic Factors that Block Optic Nerve Regeneration

There are many inhibitory molecules to axon growth that are expressed by glial scar and myelin. The proteins associated to myelin that exerts inhibition to axonal growth are the oligodendrocyte-myelin glycoprotein (Omgp), myelin glycoprotein (MAG), Nogo A, ephrins, semaphorins, and lipid sulfatides [37–45]. They bind to a receptor complex formed by Nogo receptor-NgR and LINGO1 and either low-affinity trophic factor receptor p75 and TROY [46–48]. The interaction of the ligands with the receptor complex activates a small GTPase RhoA which promotes growth cone collapse [49–52].

The glial scar at the lesion site also expresses inhibitory molecules, the chondroitin- and keratin sulfate proteoglycan (CSPG and KSPG). They bind to the protein tyrosine phosphatase receptor (PTP) sigma [53], transmembrane leukocyte common antigen-related phosphatase (LAR) [54], and two NgR isotypes [55] and like the myelin proteins they also act through the GTPase RhoA [56, 57].

12.5 Changes in RGCs' Intrinsic Growth State During Development

During rodents' development, RGCs present a shift in their intrinsic growth capacity. They go from a state of powerful growth during embryonic ages, where cells can extend neurites after an injury, to a state of no growth; this latter happens in very early postnatal periods. The changes seen at this time coincide with the period where amacrine cells establish contact with RGCs [44]. This cell–surface interaction triggers the changes in the intrinsic growth capacity of the cells. At this stage of development, there is a change in gene profile expression and regulation of transcriptional factors. Transcriptional factor of the KLF family is one example, KLF4 is upregulated at the same time as RGCs lose their intrinsic capacity, and deletion of

the *klf4* gene in adult mice promotes moderate regeneration [58]. On the other hand, members of the same family, the KLF6 and -7, are downregulated. Reports from experiments done on zebrafish show that these latter are important for optic nerve regeneration [59, 60]. Other molecules also decrease their expression during development and some of them are related to regulation of transcriptional factors, for instance the histone acetyltransferase p300, a transcriptional co-activator [61].

12.6 Cell Signaling Pathways and RGCs' Regeneration

There are several signaling pathways involved in optic nerve regeneration. As mentioned previously in this chapter, oncomodulin is a potent neurotrophic factor and it was shown that it can exert its effect by activation of at least three distinct signaling pathways, the PI3K/Akt, Jak/STAT, and MAPK [26]. When blocking each one of these pathways, no decrease on axonal regeneration is observed. However, when blocking all of them simultaneously, the inflammation-induced regeneration is abolished. This result demonstrated that some signaling pathways act synergistically, yet whether oncomodulin was able to fully activate each one of these pathways remained unknown [26]. Few years later Park and colleagues showed that deletion of *pten* (phosphatase and tensin homolog) alone, from RGCs in mice, was sufficient to stimulate a strong axonal regeneration after optic nerve injury. PTEN is a suppressor of PI3K and deletion of this phosphatase leads to activation of mTOR pathway [13]. Later on, Kurimoto and colleagues combined deletion of PTEN together with intraocular inflammation and found a tenfold increase on axonal regeneration compared to PTEN deletion alone. These results implied that oncomodulin itself cannot fully activate the PI3K pathway, and that PTEN deletion together with intraocular inflammation caused an additional activation of the PI3K pathway. In this work, Kurimoto and colleagues showed long-distance regeneration of optic nerve axons, with some fibers reaching the chiasm and very few entering the dorsal lateral geniculate nucleus in mice [29].

SOCS3 is another important molecule and is a suppressor of Jak/STAT pathway. The trophic factor CNTF acts through this pathway but after optic nerve injury the levels of SOCS3 increase and Jak/STAT is blocked, preventing any effect of endogenous or exogenous CNTF. Double deletion of the genes that encodes SOCS3 and PTEN demonstrated fibers reaching the chiasm but the amount of axons in the optic nerve decreased along the optic nerve and over time after injury [62].

The DLK is also essential for RGC response to stress and it is upregulated right after the lesion. As mentioned in a previous section in this chapter it has a controversial role, being both proapoptotic and proregenerative following nerve injury. Deleting *dlk* affords robust neuroprotection to RGCs but blocks axon regeneration and even with double deletion of DLK and PTEN animals showed a blockage of axonal regeneration. This result suggests that DLK is essential for stimulation of axonal regeneration [12].

12.7 Combination of Therapeutic Strategies and Optic Nerve Regeneration

We have previously discussed molecules that inhibit axonal regeneration after an injury to the optic nerve, as well as signaling pathways that stimulate the intrinsic growth state of RGC. These discoveries led some groups to combine some of these therapies with the aim of getting increasingly stronger regeneration.

It is known that NgR mediates the growth-inhibiting effect of three myelin proteins, MAG, Omgp, and Nogo. Suppression of NgR activity and stimulation of RGC growth capacity increased axonal regeneration several-fold; however, when overexpressing NgR the axon regeneration is almost completely abolished [63]. Combining intraocular inflammation with inactivation of RhoA—which is a converging point to signals from different myelin and glial scar inhibitory molecules—induces RGC to regenerate their axons to levels higher than when inhibiting RhoA alone after nerve crush [21]. More recently, it was shown that deletion of *NgR1*, *NgR3*, and *RPTP* σ this latter being a chondroitin sulfate proteoglycan receptor, associated with zymosan injection had a twofold increase in regeneration when compared with deletion of the receptors alone, without the stimulation of growth potential by inflammation on RGCs [55]. Pernet and colleagues used a model in which STAT3 is constitutively active after optic nerve injury and found that this is sufficient to increase the levels of axon regeneration. However, when only stimulating this signaling pathway, axons made massive U-turns—growing axons turning back to the lesion site—suggesting defects in axons directionality and guidance. The axonal U-turns were reduced when they blocked ROCK, a key component of the myelin-associated growth inhibitors [64].

In order to promote an extensive regeneration, several groups have tried to combine different strategies that have been shown to stimulate a robust increase in axonal regeneration and cell survival. Inflammation-induced regeneration by intraocular injection of zymosan and cAMP stimulates at least three distinct pathways [26]; if additional to that *pten* gene is deleted there is an increase on the levels of axon regeneration and cell survival (Fig. 12.2) [29]. If one can maintain the concentration of oncomodulin at appropriate levels, RGCs can keep their growth potential active for up to 6 weeks, a period where some fibers reach the chiasm and very few can be found in the lateral geniculate nucleus (LGN) (Fig. 12.3) [29]. Hence, our group went further, trying to stimulate the intrinsic growth state of RGCs for longer time. We found that with appropriate stimulation the cells can keep their axonal elongation for up to 12 weeks [65]. More fibers can reenter the brain and reach nuclei involved in imaging formation as well as the ones that are not involved with imaging formation. Analysis by electron microscopy demonstrated axons in different stages of regeneration, some axons with no myelin, others with thin myelin, and also axons with normal aspect—intact axoplasm and myelin sheath with normal thickness. Regenerating axons reinnervated the suprachiasmatic nucleus (SCN), dorsal lateral geniculate nucleus (DLG), olivary pretectal nucleus (OPT), medial terminal nucleus

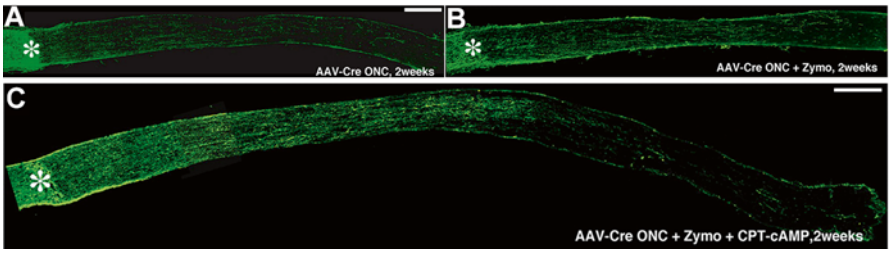

Fig. 12.2 Optic nerve regeneration induced by combinatorial treatment in mice. GAP-43 staining 2 weeks after nerve injury in mature mice. (**a**) Regeneration after *pten* deletion. (**b**) Zymosan (Zym) and *pten* deletion induces a moderate amount of regeneration. (**c**) The combination of Zymosan, cAMP elevation, and *pten* deletion induces much greater regeneration than any of these alone. Scale bar: 200 μm. [29]

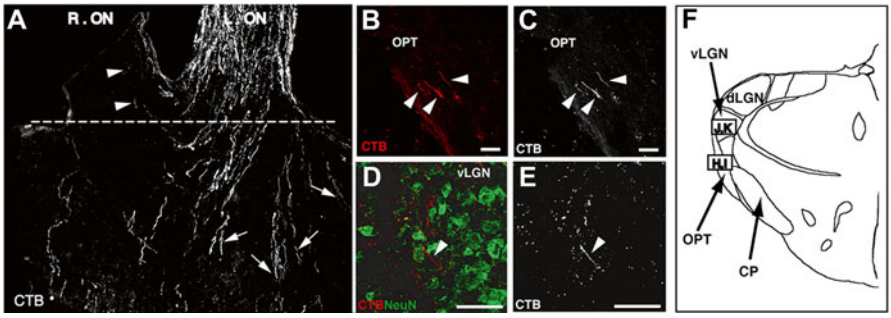

Fig. 12.3 Synergistic interactions induced by combinatorial treatment lead to long-distance regeneration after 6 weeks. Regenerating axons from RGCs exposed to deletion of *pten* gene + intraocular inflammation + CPT-cAMP. Mice received intraocular injections of cholera toxin B fragment (CTB) 4 days before being prepared for histology. The chiasm was immunostained for CTB. (**a**) Regenerating axons in the optic chiasm. *Arrows* point to regenerating axons that extend into the thalamus; *arrowheads* show axons growing into the contralateral optic nerve. (**b**, **c**) Extension of CTB-labeled axons into the thalamus. Sections were double stained to detect CTB in regenerating axons (*red*) and NeuN to visualize neurons (*green*). Panels (**b**, **d**) show double staining, whereas (**c**, **e**) show the axons alone at high contrast. Some axons can be seen in the contralateral optic tract (**b**, **c**) and ventral lateral geniculate nucleus (**d**, **e**). *Arrowheads* indicate CTB-labeled axons. (**f**) Schematic drawing through the thalamus showing positions of labeled axons. dLGN, dorsal lateral geniculate nucleus; vLGN, ventral lateral geniculate nucleus; *OPT* tract, *Ch* chiasm, *CP* cerebral peduncle. Scale bars in (**b–e**) 50 μm [29]

(MTN), and the superior colliculus (SC) (Fig. 12.4) [66]. To determine whether the reinnervation of central targets would have functional consequences, animals were tested for innate visual behaviors. We observed that some visual reflexes were partially recovered, such as circadian photoentrainment, optomotor reflex—a visual guided behavior and depth perception (Fig. 12.5) [65]. These results are extremely exciting to the field of optic nerve regeneration and they point out to a possible route toward translation of molecular biology into clinical studies.

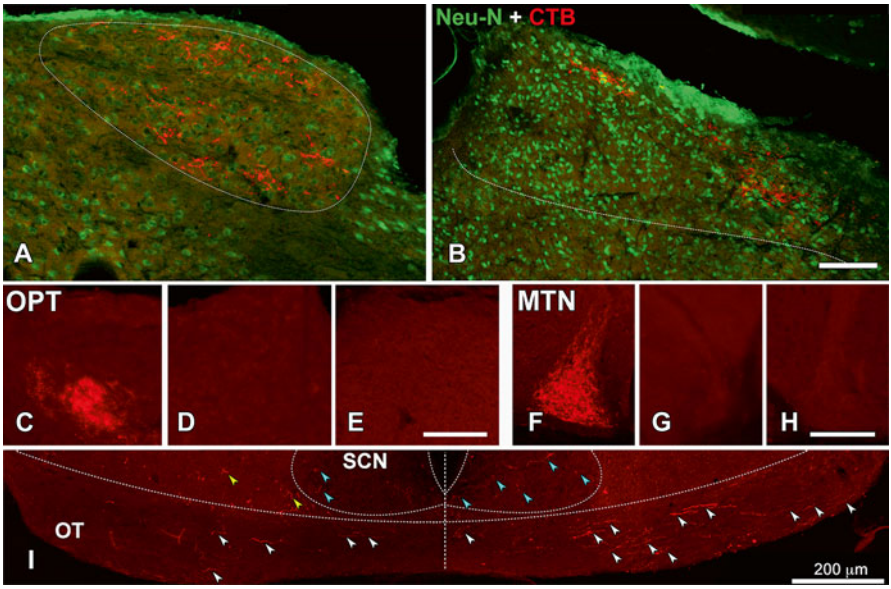

Fig. 12.4 Reinnervation of visual nuclei. Reinnervation of central visual targets 10 weeks after optic nerve injury, including the dorsal lateral geniculate nucleus (dLGN, **a**) and superior colliculus (SC, **b**), both of which are stained for CTB (*red*) to visualize regenerating axons and Neu-N (*green*). Regenerating axon terminals stained for CTB in the Olivary pretectal nucleus (OPT, **c**) and medial terminal nucleus (MTN, **f**). No fibers were seen on the side ipsilateral to the regenerating optic nerve (**d**, **g**) or in control animals with incomplete regeneration (**e**, **h**). Panel (**i**), CTB+ axons in the optic chiasm. Note axons coursing in the optic tract on the right side (contralateral to the regenerating optic nerve) and a smaller number on the left side (*white arrowheads*). Some CTB+ profiles are seen within the SCN bilaterally (*blue arrowheads*) and some are outside this area (*yellow arrowheads*). Scale bars: (**a**–**h**), 100 µm [65, 66]. Reproduced with permission from PNAS

Regeneration and functional recovery in other CNS area were previously shown. Researchers demonstrataded that hippocampal neurons in adult animals formed functional synapses onto appropriate targets [67–69]. Our work has added a new insight in the fild of CNS regeneration. [67–69]. However, another group used the same combinatorial treatment that de Lima and colleagues have used but they fail to show the same degree of central reinnervation [70], and this might be explained by the fact that Lou and collaborators have not adopted the same methods described in the former work. However, finding out the central issue for these controversial results is fundamental.

Another group has shown long-distance regeneration using another combination of therapy. They showed that double deletion of *pten* and *socs3* can stimulate long-distance regeneration and axons could reach the optic chiasm, although very few fibers reentered the brain at the level of the SCN nucleus. This type of stimulation induced the expression of many genes related to axon regeneration as well as maintained this repertoire of gene expression profile at physiological levels after axon injury [62].

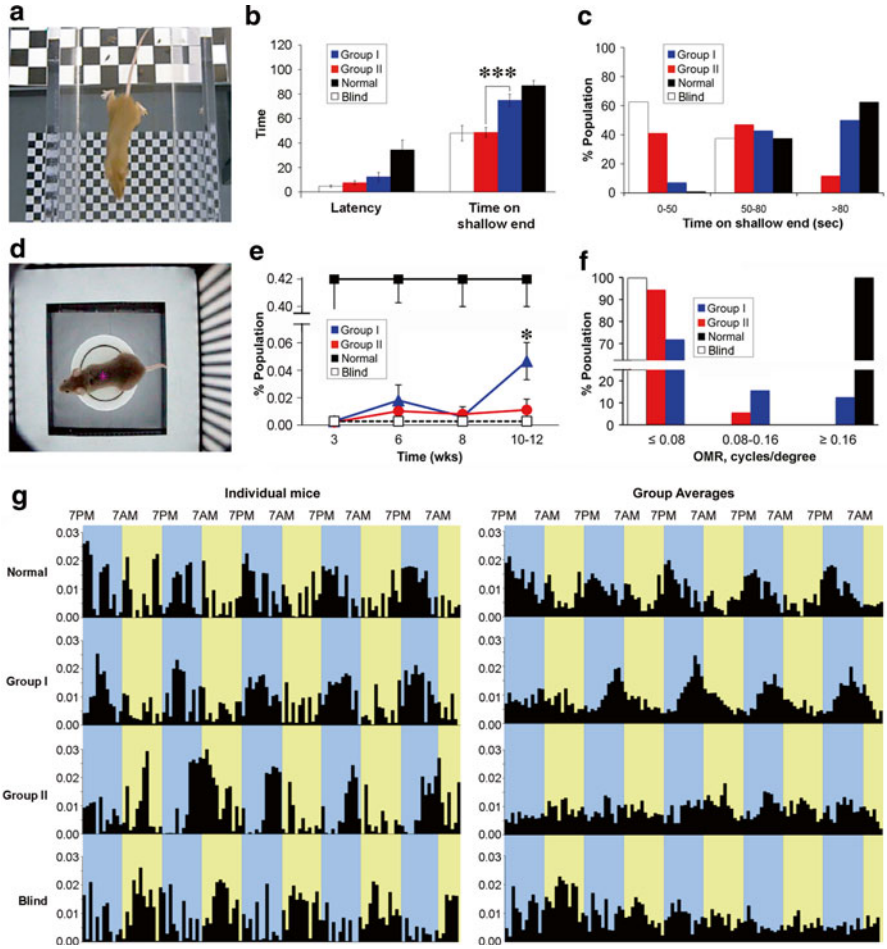

Fig. 12.5 Partial recovery of function. Tests for three visual responses in normal mice (*black bars*) and in mice with optic nerve injury and the *pten* gene deleted (Group I, *blue bars*) or present (Group II, *red bars*). Mice in Groups I and II both received intraocular inflammation combined with CPT-cAMP. (**a**) Visual cliff apparatus used to evaluate depth perception. (**b**) *Left*: Mice with optic nerve damage show a tendency to step off the shallow end faster than normal animals. *Right*: Total time spent on shallow end. Group I mice, like normal controls, show a preference to return to the shallow end. ***$P<0.01$. (**c**) Histogram showing distribution of population from each group and the time spent on shallow end. (**d**) Apparatus used to evaluate optomotor response (OMR). (**e**) Average OMR (response threshold, cycles/degree) as a function of time. Note improvements in Group I. (**f**) Frequency distribution of the OMR. The *y*-axes in *e* and *f* are discontinuous. (**g**) Circadian photoentrainment: *Left*: percent of overall activity in 1-h bins for individual mice and; *Right*: group averages. Mice were maintained on a continuous cycle of lights on at 7 AM and off at 7 PM prior to testing and for the first 2½ days in the activity monitor. The light cycle was set back 6 h on day 3. Error bars represent SEM [65, 66]. Reproduced with permission from PNAS

12.8 Future Perspective

Although many biological intrinsic and extrinsic mechanisms related to axonal regeneration as well as neuron survival have been discovered, there is still many to be made to achieve complete visual recovery in rodents. It is well established that multiple pathways are involved in axon regeneration, and at least mTOR pathway can stimulate both regeneration and survival. However, it is still unknown which are other possible pathways that can protect neurons from dying. Discovering how to improve the levels of RGC survival would be essential to test combination of therapies that could get even more regeneration and reconnection to brain targets. Another important issue that has to be considered is the investigation of molecules responsible for axon guidance of regenerating neurons, how they are organized in the adult CNS, and how they influence the appropriate targeting during regeneration. After advancing on these questions it will still be necessary to optimize the methods to try to translate them into clinical trials, this way we could have effective treatments that could prevent or recover visual loss.

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Chapter 13
Bionic Eyes: Vision Restoration Through Electronic or Photovoltaic Stimulation

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Contents

Abbreviations

ADLs	Activities of daily living
AMD	Age-related macular degeneration
ASR	Artificial silicon retina
BaLM	Basic assessment of light and motion
BDNF	Brain-derived neurotrophic factors
BVA	Bionic vision Australia

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A. Pébay (ed.), *Regenerative Biology of the Eye*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4939-0787-8_13,

LGN	Lateral geniculate nucleus
QoL	Quality of life
RCS	Royal College Surgeon
RP	Retinitis pigmentosa

13.1 Introduction

Blindness is one of the most feared disabilities [1], having a significant and debilitating impact on both an individual's quality of life (QoL) and the wider social economy. Whilst there are existing treatments for some common causes of blindness, such as cataracts, many diseases remain untreatable with blindness or severe vision loss still an inevitable outcome. For example, it is estimated that retinitis pigmentosa (RP) affects around 1.5 million people worldwide, making it the leading genetic cause of inherited blindness [2]. There is currently no treatment for RP. Age-related macular degeneration (AMD) is another example of a common cause of irreversible vision loss in developed countries, affecting millions of people, predominantly in the elderly population [3, 4]. Whilst there have been tremendous advances in treating the earlier stages of the neovascular form of advanced AMD, there is no treatment for people who have already lost their vision.

Whilst a number of vision restoration techniques are being developed, such as stem cells or gene therapy, these remain in their early phases of development and may not benefit people with end-stage disease. Visual prostheses (bionic eyes) offer another approach for restoring a basic level of functional vision. Visual prostheses work to convert visual information into electrical impulses, in a similar way that the cochlear implants have worked to restore hearing to the deaf. Research is underway worldwide into various forms of visual prostheses, such as retinal (suprachoroidal, subretinal and epiretinal implants), optic nerve cuffs and cortical implants. The majority of groups, including our own—Bionic Vision Australia (BVA)—are investigating the retinal approach.

This chapter will cover the current theory and progress in the field of visual prostheses, and touch on the future possibilities of the technology. Whilst the realities of prosthetic vision in 2013 are still basic, the potential for vision restoration in the future is promising.

13.2 History of Visual Prosthetics

Ever since the eighteenth century, scientists have sought to restore vision with the use of electrical stimulation of the eye or visual cortex [5]. In the 1920s and 1930s, the German neurosurgeon Foerster discovered that direct electrical stimulation of the visual cortex caused blind patients to detect a spot of light, known as a phosphine [6]. He also proved that the location of the detected phosphenes moved when

different parts of the cortex were stimulated [6]. In 1931 Krause and Schum electrically stimulated a region of hemianopia in a patient that suffered a gunshot wound to the left optic radiation 8 years previously, showing that phosphenes could be generated even after years of cortical deprivation [7]. However, these early devices had inherently poor resolution, were constrained by the size restrictions that technology at the time allowed and often had severe adverse events once implanted [8].

There was little further research in this area for over 30 years, until microtechnology developed further and the size of such devices could be miniaturised, whilst maintaining their required levels of power. In the 1960s, Australian inventor and radio engineer Graham Tassicker patented a photosensitive selenium cell that could be placed subretinally to evoke visual phosphenes [9]. This discovery reinvigorated the visual prosthetic research field, and groups again considered the idea of using a cortical visual prosthesis to restore vision to the blind.

Early studies, including those by Brindley and Dobelle, investigated the number of cortical electrodes that would be necessary to evoke phosphenes, both theoretically [10, 11] and in acute and chronic stimulation studies [12–14]. These early studies also showed variations in the appearance of the evoked phosphenes, from stationary to flickering, simple white to multi-coloured complexes [15]. However, technological advances in cortical visual implant research have been limited by capabilities in resolution and long-term stability of cortical electrodes. There have been some recent developments in cortical implant research, with improvements in electrode configuration and material development that may allow clinical trials to begin in the foreseeable future [16–21].

For now, most progress has been made on retinally located implants. Until the 1970s, this was not a viable option due to the invasiveness of retinal surgery, which led to a high risk of adverse effects. However, since the introduction of the more sophisticated surgical techniques [22–25], experienced retinal surgeons are now able to place small electrodes behind the retina [26] or attach the device onto the retina (epiretinal) [27].

13.3 Background Theory of Prosthetic Vision

While the theory behind visual prostheses has been proven by successful clinical trials in the USA and Germany [26, 27], it is still undeniable that the development of a bionic eye is one of the most difficult technological challenges that biomedical engineering has faced to date. To give an indication of the complexity of the procedure, we can consider the cochlear implant, which has restored hearing to thousands of deaf people worldwide. In humans, the auditory system relies on approximately 15,000 hair cells in the cochlea and a similar number of sensory neurons [28]. Successful cochlear implants have been designed to transmit useful levels of sound with 16–20 electrodes [28], and after over 30 years of development the technology is now well accepted and successful. In terms of vision, the technological requirements are many magnitudes higher, with the analogy often used that it is like the difference between

making a radio and a television. In healthy human retina, there are approximately 120 million retinal photoreceptors and 1.2 million optic nerve fibres [28]. This means that if the same ratios apply for electrodes needed per quantum of neurons as that used for cochlear implants, a retinal prosthesis would need to have at least 1,000 electrodes for a similar level of performance [28]. It is estimated that several hundreds to thousands of high-density electrodes would be required for the restoration of sight to enable adequate visual acuity to read a visual acuity chart [29–31].

The retina is an extremely complex tissue, providing extensive signal pre-processing before the signal is passed to the brain. One of the other challenges is that in retinal degenerations there is significant reorganisation of local retinal networks [32, 33], such that stimulation of a particular region of retina may not correlate directly to a percept of vision in the area of visual field that would normally be expected. Other changes in the eye also need to be considered, such as changes to the retinal blood supply. We have shown that in RP the choroidal blood vessel layer becomes thinner, correlating with the duration of disease [34]. This choroidal atrophy and resulting change in physiological properties need to be considered when developing a retinal prosthetic implant.

The challenges of prosthetic vision are many, but the theory is sound, and it is a viable option. For a retinal prosthetic implant to be effective, sufficient inner retinal neurons must remain despite severe photoreceptor cell loss in degenerative retinal disease. A frequency-domain optical coherence tomography study by Hood et al. revealed that this is the case in RP, with no significant difference between the thickness of inner nuclear and retinal ganglion cell layers in RP patients and controls [35]. Post-mortem morphometric analysis of the retina of patients with end-stage RP has also shown relative sparing of the inner retinal layers, with a higher percentage of inner nuclear cells (78.4 %) and ganglion cells (29.7 %) remaining than photoreceptors (4.9 %) [36–38]. This preservation of inner retinal neurons is also seen in both atrophic [39] and neovascular [40] forms of AMD. Hence, theoretically, retinal micro-electronic implants should allow some level of vision in patients who are blind or severely visually impaired from these two conditions, as there is limited trans-synaptic neurodegeneration [41]. In other words, the retinal prosthesis works by bypassing the dead photoreceptors to directly stimulate the inner retinal neurons, thus utilising the intact posterior visual pathway to transmit signals to the visual cortex.

13.4 Current Research

There are at least 30 distinct research groups worldwide that are working on visual prosthesis development at this time. Most of the groups are in the stages of testing the safety of the implantable electrode and determining the threshold for the electrical stimulation in preclinical or simulated models. A few groups have initiated human clinical trials [26, 42–44], with the Second Sight group in the USA gaining European CE mark approval in 2011 and FDA approval in 2013, and the Retina

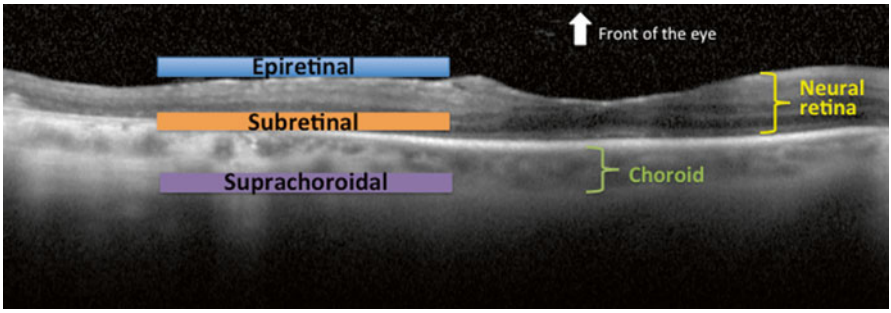

Fig. 13.1 Cross-sectional optical coherence tomography (OCT) image of the retina and choroid, showing the possible locations of retinal visual prostheses (epiretinal, subretinal and suprachoroidal) and the neural retina and choroidal layers

Implant AG group in Germany receiving CE mark approval in 2013. These approvals are a huge advance for the research field and mean that patients are now able to commercially purchase the devices in some countries.

The main components of the most prosthesis systems include a camera mounted on a pair of glasses, computer microprocessor, a battery and an electrode array for stimulation. When an image enters the camera, the video camera sends the captured image to the microprocessor for complex processing and determining the parameters (e.g. magnitude and duration) of the electrical stimulation. The processor then sends signals, either by a cable or wirelessly, to the implanted electrode array to initiate the electrical stimulation. As discussed previously, these electrode arrays may be implanted into four main areas of the visual pathway—the retina, optic nerve, lateral geniculate nucleus (LGN) or the visual cortex.

The retinal approach works by stimulating the remaining retinal neurons with an electrode array placed either on the retina (epiretinal implantation), underneath the retina (subretinal implantation) or behind the posterior vascular choroid (suprachoroidal implantation), Fig. 13.1.

13.4.1 Epiretinal Prostheses

Epiretinal devices have the electrode array attached to the inner retinal surface, with current devices using mechanical tacks to maintain device stability. Whilst this means that the device is in close proximity to the ganglion cells in the inner retina, it also means that the mechanism of attachment may potentially damage the very tissue needing to be stimulated. Mechanical tacks also present problems for the longevity and robustness of the attachment, with a higher chance of the device dislodging than in subretinal or suprachoroidal implantation. However, preclinical studies by the Second Sight group have shown the mechanical attachment of

epiretinal devices appears to have minimal effects on retinal layers [45]. There are also numerous reports of longer term human implantations of these epiretinal devices, with few cases of tack dislodgement [27, 46, 47]. As such, the epiretinal device does appear to be a viable approach for the implantation of visual prostheses.

The epiretinal approach is designed to stimulate the retinal ganglion cells directly, bypassing the processing function of the bipolar and amacrine cells that occurs in the outer retina. As such, epiretinal systems may require more image processing algorithms and complex stimulation patterns to account for this loss of retinal processing than would occur if these processes could be utilised [48]. One main advantage of the epiretinal approach over others is the ability to take advantage of the vitreous as a heat sink, allowing dissipation of heat generated in the stimulation away from the retinal tissue and hence limiting the chance for electrode-induced thermal damage to the retinal cells [49].

The most clinically advanced epiretinal prosthesis to date is the Argus II implant, which was patented by Second Sight Medical Products Inc., California in 2007 in the US, and received FDA approval for commercialisation in 2013. The Argus II implant has 60 platinum micro-electrodes, and clinical trials in 32 subjects have shown that the device can improve motion detection, mobility and object detection [27, 50]. The company's first generation device, Argus I, a 16 electrode device, has also been shown to provide improvement in patient visual perceptive tasks such as object detection, counting and discrimination and direction of object movement [42, 51]. The other two advanced epiretinal research programs are the Learning Retinal Implant, developed by Intelligent Medical Implants AG [52, 53], and the EPI-RET3 device from the EpiRET GmbH group in Germany [54]. There are other epiretinal devices in early stages of development and testing, but these have not progressed to clinical trials to date [55–57].

13.4.2 Subretinal Prostheses

The subretinal approach allows the device to be placed in the space between the retina and the choroid either via a scleral incision (ab externo surgery) or through the vitreous cavity and retina (ab interno surgery). The subretinal prosthesis is supported by the natural adherence forces that exist between the retinal pigmented epithelium and the sensory retina [58]. In this position, the bipolar and amacrine cells can be directly stimulated, taking advantage of the retinal processing which occurs in these neuronal pathways.

To date, two subretinal implants have been tested in human clinical trials. The Artificial Silicon Retina (ASR) implant was developed by the Optobionic Corporation (IL, USA) and is reliant on incident light for activation [44, 59–61]. Phase II clinical trials showed that natural incident light did not provide enough stimulation to activate the remaining retinal cells [62], and hence this device is no longer being tested or manufactured. An interesting finding was that improvements in retinal function were noted in areas further away from the implant, which could be due to a neuroprotective effect on the remaining retinal cells [63, 64].

The other subretinal implant that has been trialled in humans is that from Retina Implant AG, a German company affiliated with the University of Tübingen. The Retina Implant AG device was awarded CE Mark approval in 2013. As such, their device consists of a light sensitive 3.0×3.1 mm chip with 1,500 electrodes. This device has been trialled in 12 subjects for a month with no complications [26, 65] and showed improved object localisation and even recognition of individual letters in one subject [26]. Second stage multi-centre clinical trials are underway for this device.

The Boston Retinal Implant Project (BRIP) originally began by developing an epiretinal implant [66, 67], which was abandoned due to instability and inconsistent results [68]. They are now working on a wireless subretinal device, which is currently undergoing safety and efficacy studies in animals [69, 70]. A group from Stanford University have also developed a subretinal implant, which uses silicon photodiodes and pulsed near infrared illumination to stimulate the retinal neurons [71, 72]. Clinical trials for these devices are anticipated in the coming years.

13.4.3 Intrascleral and Suprachoroidal Prostheses

Two groups have looked at placing the electrode arrays behind the posterior vascular blood supply of the eye (choroid), which is a more surgically accessible and stable position. Such devices can either be implanted within the outer scleral wall of the eyeball (intrascleral implantation), or in the area between the choroid and the sclera (suprachoroidal implantation).

It is assumed that suprachoroidal implants will require more electrical current for stimulation than those placed in the subretinal position, because the suprachoroidal electrodes will be further away from the target ganglion cells [73]. However, it has been shown that devices placed in these positions are still able to evoke phosphenes within safe charge limits and with a good dynamic range [74–77].

A Japanese team found that electrode arrays placed in an intra-scleral pocket could evoke phosphene percepts in two patients with end-stage RP [74]. Bionic Vision Australia have shown safety and efficacy of a suprachoroidal device in both preclinical models [77, 78] and in preliminary patient testing [79, 80]. The BVA pilot study (completed in 2012–2014), used a prototype suprachoroidal implant with 30 platinum electrodes (of which 20 could be individually stimulated), and found improvements in visual function with device on, in addition to an excellent safety profile.

13.4.4 Optic Nerve Prostheses

There are two main forms of optic nerve visual prosthesis: self-sizing spiral cuff electrodes that wrap around the nerve [81–83] and insertion of multiple penetrating electrodes into the nerve and optic disc [84–88]. A cuff electrode activates

numerous optic nerve fibres at once (giving the perception of large phosphenes) whilst the penetrating micro-electrode is targeted with more discrete activation.

Subjects implanted with a spiral cuff electrode developed by Veraart and colleagues were able to localise and discriminate basic objects [89–91] and had basic pattern recognition ability [92]. The alternative penetrating electrodes have had less functional vision success. Whilst it has been shown that intra-orbital optic nerve stimulation with penetrating electrodes can evoke cortical responses in rabbits [87] and can generate perception of phosphenes in humans [84], patients implanted with these devices have not been able to use these phosphenes to improve their functional vision and were unable to detect or localise objects with the device.

13.4.5 Lateral Geniculate Nucleus Prostheses

LGN visual prostheses are a far less common approach but are in early experimental stages [93–95] and target the sensory neurons that project directly to the visual cortex. There has been little recent research into these devices.

13.4.6 Cortical Prostheses

Stimulation of the visual cortex to produce phosphenes [6] was the historical progenitor of this research field. The original studies used extra-cortical stimulation, which required high levels of energy and often lead to adverse effects such as discomfort or epilepsy [13, 14]. The risk level associated with these experiments decreased when Schmidt invented an intra-cortical electrode, which allowed lower levels of current to be used in the stimulation protocol [96].

The advantage of an optic nerve, LGN or cortical prosthesis over a retinal prosthesis is that these positions for electrical stimulation do not rely upon intact retinal ganglion cells [17]. As such, they may be a treatment option for other causes of vision loss, such as glaucoma and trauma.

Modern cortical implants work by placing penetrating electrodes directly in the primary visual cortex [17]. Current research projects in cortical prostheses include the Utah Electrode Array (UT, USA) [21, 97], the Illinois Intra-cortical Visual Prosthesis Project (IL, USA) [19, 20, 98] and the Monash Vision Group “Gennaris” bionic eye (Melbourne, VIC, Australia) [99].

13.4.7 BrainPort™ Sensory Substitution Prostheses

There is one other alternative visual prosthesis currently being trialled in humans, which does not require any direct stimulation to the visual pathway. The BrainPort™ is a sensory substitution device which allows the blind to perceive their environment by means of a glasses-mounted camera, paired with a 400 electrode tactile array placed on the tongue [100, 101]. The device has been shown to produce form vision perception, and also activates the visual cortex simultaneously, providing evidence for cortical plasticity [102].

13.5 Visual Function Testing Pre- and Post-prosthesis Implantation

One of the biggest challenges facing all vision restoration clinical trials (including stem cells and gene therapy) is the development of sensitive and specific outcome measures. The majority of the patients will have bare light perception or no light perception level vision at baseline and, as such, standard visual acuity, visual field and visual function tests are not appropriate to define the baseline function. In addition, these standard tests were not designed to measure small increments in performance and so are unable to detect the improvements seen in these early vision restoration studies. At present, different groups have developed different testing protocols, but it is imperative that through collaboration a standardised protocol can be agreed upon [103].

Given the small number of clinical trials for vision restoration to date, many of these visual function outcome protocols have been developed using simulations, using computer programming to convert visual scenes into phosphene maps [104, 105]. These studies have allowed researchers to develop a sense of how best to measure and record visual performance, as well as give some indication of what performance might be like at different levels of phosphene generation [106, 107]. Such simulated phosphene studies have also enabled researchers to develop recommendations for required rehabilitation regimes [108].

Quantitative tests of visual acuity for those with extremely low vision have been proposed [26, 109–111]. These tests include the Basic Assessment of Light and Motion (BaLM), which allows determination of visual function from a simple determination of black versus white through to motion discrimination [109]. The most widely tested visual prosthesis, to date the epiretinal Argus I device, showed variable results when measuring visual acuity, but some patients were able to be tested using sinusoidal grating acuity measures [112, 113]. To date, no implanted devices have allowed subjects to read a standard Snellen or logMAR visual acuity chart in a normal manner.

Many people who have lost all vision comment that they would like to regain some orientation and mobility skills, in order to increase independent travel [114]. To develop appropriate navigation and mobility tests, researchers have used simulated prosthetic vision setups [115], which can be adapted into real world situations when needed.

The aim of a visual prosthesis is to improve the QoL for a person with extremely low or no vision. This can be assessed through a range of measures which fall under the term of "functional vision assessment", including assessments using activities of daily living (ADLs) as well as orientation and mobility skills. There have been reports of subjects with retinal prosthetic implants being able to correctly describe and name objects like a fork or knife on a table, geometric patterns and different fruits [26].

Another important aspect to consider when assessing the success of visual prosthetic devices is patient expectations and needs. Focus groups are a good way to understand more about what people with extremely poor vision would like from an artificial vision device. A series of focus groups were held in Melbourne which revealed a wide range of needs and desires from the participants, with some desiring a return to full visual ability (including the ability to drive a car), and others happy to gain a small increase in independence and mobility [114]. Patient perspectives and expectations are vital when considering QoL outcomes, and there is a need for standardised, validated QoL surveys for visual prosthesis clinical trials.

13.6 Using Visual Prostheses for Preservation of Photoreceptors

Currently, there is no effective treatment to promote the survival of the photoreceptors in retinal degenerative diseases such as RP. In recent years, there has been an increasing interest in using micro-current stimulation for improving the survival of the photoreceptors in retinal degeneration. This field of research was in part stimulated by an accidental discovery that low level electrical stimulation delivered by an ASR microchip implanted in the subretinal space resulted in improvements in visual function in patients with RP [44]. The improvement in visual function (both in visual acuity and visual field) first developed from several weeks to months after implantation and persisted for up to 3.5 years of follow-up [44]. Similar preservation of residual hearing has been noted in patients with cochlear implants [116–118], giving support to the idea that visual prostheses may be an option for preservation of photoreceptors.

The effect of electrical stimulation on promoting the survival of photoreceptors and preserving retinal function has been noted in retinal degeneration models including transgenic rabbits [119] and the Royal College Surgeon (RCS) rats [120, 121]. In these clinical and experimental studies, stimulation was performed by injecting current through electrodes, which were either placed on the surface of the eye (known as transcorneal electrical stimulation) or within the retina (known as subretinal electrical stimulation).

It is believed that electrical stimulation induces the production of endogenous neutrotrophic factors which promote the survival of the retinal neurons [122, 123]. Stimulation-induced up-regulation of the mRNA of various neurotrophic factors [120] and the production of brain-derived neuroprotective factor (BDNF) in cultured Müller cells have been reported [124]. Another possible mechanism for the stimulation-induced neuroprotection is an increase in chorioretinal blood circulation [125].

Electrical stimulation has been shown to improve the visual function of patients with retinal artery occlusion [126, 127].

A recent prospective randomised clinical trial in a small cohort of RP patients showed a positive trend of improvement in visual field and some components of the electroretinogram [128]. This is an interesting field of research, which has the potential to increase the applicability of electrical stimulation with visual prostheses. However, these findings need to be confirmed in future studies with larger sample size and longer follow-up duration.

13.7 Other Future Ambitions

Currently implants are tested in people who were born with some functional vision [129], so that there will have been development of the visual system and formation of the trans-synaptic connections which are believed to be important for the artificial stimulation of neurons to be successful [130]. It has been shown that if blindness onsets before the age of six, the human visual system is abnormally organised and so potentially would have very different responses to stimulation compared to others whose visual system was fully developed before blindness ensued [131]. However, in the future implantation in young children may be an option due to the plasticity of neuronal circuitry in early childhood [28]. These children may potentially have, due to their brain plasticity, the greatest potential for effective prosthetic vision, in an analogous manner to the success of children who receive cochlear implants at an early age. This research would most likely occur after the long-term efficacy and safety of the device was assured.

13.8 Conclusion

The idea of regaining sight after years of blindness is inspiring to all, but it is important to realise that the visual prostheses will not help all patients and that this restored artificial vision will not be the same as normal sight. Retinal implants require preservation of the inner retinal neurons [41], which excludes a large group of patients who would require stimulation further down the visual pathway such as through the optic nerve, LGN or cortical prostheses. To date, these devices have not progressed to the stage of clinical trials.

The advantage with using an electrode array to stimulate the visual pathway in a visual prosthesis, as opposed to photovoltaic diodes, is that computer image processing can be implemented to optimise visual outcomes from the devices [132]. As these processing algorithms are rapidly improving, visual prostheses have significant potential for vision restoration in the future.

Whilst the use of visual prostheses is still an emerging technology, the future of these devices is promising and brings hope for people with profound vision loss.

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Chapter 14
Stem Cell-Derived RPE Transplantation for Age-Related Macular Degeneration: Experimental Studies to Improve Transplant Survival and Differentiation

Ilene K. Sugino, Qian Sun, Noounanong Cheewatrakoolpong, Christopher Malcuit, and Marco A. Zarbin

Contents

Abbreviations

AMD	Age-related macular degeneration
BCEC	Bovine corneal endothelial cell
BDNF	Brain-derived neurotrophic factor
BM	Bruch's membrane
CM	Conditioned medium
ECM	Extracellular matrix
fREPE	Human fetal retinal pigment epithelium
hES-RPE	Human embryonic stem cell-derived RPE
IGFBP3	Insulin growth factor binding protein-3
NGF	Nerve growth factor

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A. Pébay (ed.), *Regenerative Biology of the Eye*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4939-0787-8_14,

PDGF	Platelet-derived growth factor
PEDF	Pigment epithelium-derived factor
RPE	Retinal pigment epithelium
TGFβ-2	Transforming growth factor beta-2
TSP2	Thrombospondin-2
VEGF	Vascular endothelial growth factor

14.1 Introduction

Although a number of treatments are under study, at this time no proven treatment options exist for patients with geographic atrophy, an advanced form of age-related macular degeneration (AMD) [1]. For patients with extensive drusen and visual loss or geographic atrophy threatening the fovea, retinal pigment epithelium (RPE) transplants might prevent central vision loss through replacement of dysfunctional or dead RPE cells. RPE transplantation also might preserve or restore vision in patients with related conditions in which dysfunctional RPE cause vision loss, e.g., Stargardt disease. Cell transplantation for AMD has been undertaken using a number of cell types and preparations, including fetal and adult RPE (autologous and allogeneic), translocated autologous choroid/RPE, and autologous iris pigment epithelium (IPE) [2, 3]. In principle, transplantation of autologous RPE and IPE has no risk of immune rejection. Unfortunately, RPE from older donors do not behave as robustly as those from young donors [4–7]. In addition, autologous RPE transplants may carry AMD-related gene defects [8–10] or modifications caused by aging. Finally, older RPE may not have the ability to perform all the functions necessary to maintain the photoreceptors [11]. Fetal human RPE exhibit morphologic abnormalities after 5–6 passages, which severely limits their utility as a "universal" donor source [12]. Embryonic (ESCs) or induced pluripotent stem cells (iPSCs) can undergo large-scale expansion, assuring an abundant supply of well characterized, pathogen-free cells that can be manufactured in a manner compatible with clinical practice [13, 14]. Genetic analysis shows human embryonic stem cell-derived RPE (hES-RPE) are similar to in situ RPE [15]. In addition, they phagocytose outer segments and rescue photoreceptors in the Royal College of Surgeons (RCS) rat [16–18]. iPSCs also rescue the retina in RCS rats [14]. RPE can be derived from human embryonic stem cells (hES-RPE) in a manner that does not cause embryo destruction [19]. Manipulation of stem cell-derived RPE in culture provides an opportunity to optimize their ability to attach and survive on aged or diseased Bruch's membrane (BM) and to minimize rejection [13]. The advantages and limitations of stem cell therapy for AMD and other degenerative retinal disease have been reviewed in detail [20]. In this chapter, we will review preclinical studies that are focused on improving stem cell-derived RPE survival on aged and AMD BM. We have compared the attachment and survival of hES-RPE of different degrees of pigmentation on BM with cultured human fetal RPE (fRPE) whose behavior has been characterized previously on aged and AMD BM [21, 22].

Using an organ culture model of RPE attachment to human BM, we found that hES-RPE survival on AMD BM is poor [22]. With the addition of exogenous extracellular matrix (ECM) [23] or conditioned medium, however, survival and differentiation can be improved substantially [24].

14.2 Embryonic Stem Cell-Derived RPE Survival and Neurotrophic Factor Secretion on Aged Bruch's Membrane

After 21 days in organ culture on aged submacular human BM, nuclear densities of fRPE on submacular explants ranged from 0 to 29.69 nuclei per millimeter of BM (mean nuclear density ±SEM, 11.43 ± 1.89; Fig. 14.1) [22]. By contrast, in cell culture dishes, fRPE exhibit a nuclear density of approximately 45 nuclei/mm at

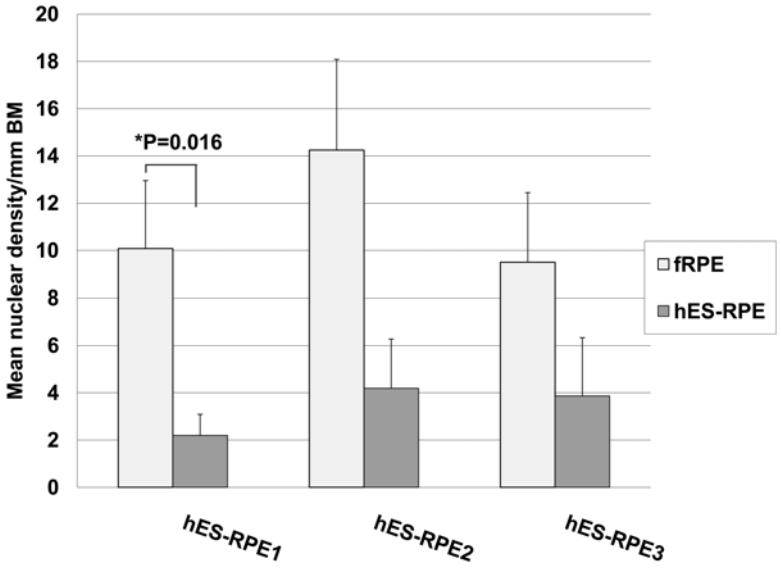

Fig. 14.1 Nuclear density of paired submacular Bruch's membrane explants seeded with fRPE or hES-RPE of different degrees of pigmentation (hES-RPE1, $N=8$; hES-RPE2, $N=8$; hES-RPE3, $N=6$; fRPE, $N=22$) on submacular Bruch's membrane at day 21 after seeding. fRPE survival was significantly greater than hES-RPE1 seeded on fellow eye explants ($^*P<0.05$). Fetal RPE nuclear densities were not significantly different from those of hES-RPE2 and hES-RPE3 seeded on fellow eye explants ($P>0.05$). hES-RPE1 (cultured for 6 weeks after harvest) exhibited little pigmentation and had fibroblastic morphology. hES-RPE2 (cultured for 9 weeks after harvest) were harvested at a later time, when cells exhibited epithelioid morphology and more than half the cells exhibited pigmentation. hES-RPE3 (cultured for 10–11 weeks after harvest) exhibited morphology similar to hES-RPE2 but more than 85 % of the cells had pigmentation. Reproduced with permission from Sugino et al. [22]

day 14 in culture, and in situ RPE in aged donors exhibit a nuclear density of approximately 30 nuclei/mm on BM for donors ≥70 years of age [21]. Different batches of hES-RPE behaved somewhat differently on BM after 21-day culture. Fetal RPE nuclear density on aged submacular BM, for example, was significantly higher than fellow submacular explants seeded with lightly pigmented hES-RPE (hES-RPE1) (Fig. 14.1; $P=0.016$; Wilcoxon signed rank test), but there was no significant difference compared with two other batches of hES-RPE, each of which exhibited increasing degrees of pigmentation [22]. Location of the BM explant (equatorial or submacular) was not associated with probability of survival of fRPE or any of the preparations of hES-RPE [22].

The presence of submacular drusen seemed to be associated with poor hES-RPE survival in the BM organ culture assay. The batches of hES-RPE tested exhibited only limited submacular BM resurfacing regardless of the extent of submacular pathology. Human fetal RPE showed limited resurfacing of three of four BM explants that exhibited substantial basal linear deposit and showed variable resurfacing of four of six BM explants with small (hard) drusen (see Table 14.1). hES-RPE with low nuclear densities on submacular BM generally appeared as small patches of cells or single cells, often not well spread and often appearing damaged with membrane holes, apoptotic blebs, or loss of cytoplasm. Explants that showed the most resurfacing by hES-RPE (i.e., seeded with hES-RPE2 or hES-RPE3 cells) exhibited incomplete resurfacing by patches of cells with highly variable morphology (Fig. 14.2). Explants resurfaced by fRPE were partially resurfaced by cells often appearing better spread and attached to BM than explants with hES-RPE. By comparison, with increasing resurfacing, fRPE formed confluent patches. However, morphology was quite variable, even within the same explant, ranging from small compact cells with short apical processes to extremely large flat cells (Fig. 14.3). Vacuoles, while present in some fRPE, were not as plentiful as in hES-RPE. Analysis of integrin expression by these cells did not reveal differences that one might correlate with differences in their behavior on submacular human BM [22]. To determine whether hES-RPE secretion of selected proteins after culture on BM is similar to that of fRPE, conditioned media above BM explants were analyzed after 21 days in organ culture [22]. On submacular BM explants, hES-RPE seemed to secrete nerve growth factor (NGF), insulin growth factor binding protein-3 (IGFBP3), pigment epithelium-derived factor (PEDF), thrombospondin-2 (TSP2), and transforming growth factor beta-2 (TGFβ-2), while fRPE seemed to secrete these proteins (except TSP2) as well as brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) (Fig. 14.4) [22].

In summary, in organ culture experiments, fRPE and hES-RPE survival was impaired on aged and AMD human submacular BM (although fRPE exhibited relatively better survival than hES-RPE). One interpretation of these results is that aged and AMD BM will not support transplants of suspensions of healthy RPE. The long-term results of RPE transplantation in most patients with AMD (both atrophic and neovascular forms) are consistent with this interpretation (see review by da Cruz et al. [25]). Despite these results, growth factor secretion by residual hES-RPE

Table 14.1 Human donor eye information and resulting nuclear density of human embryonic stem cell-derived RPE (hES-RPE) or fetal RPE (fRPE) on submacular Bruch's membrane explants 21 days after seeding

Age	D:P	D:R	COD	Submacular pathology, hES-RPE explant	Submacular pathology, fRPE explant	hES-RPE ND ± SEM[a]	fRPE ND ± SEM
59	5:03	32:48	Respiratory failure	None noted	None noted	12.84 ± 0.28[3]	15.27 ± 0.31
67	6:49	45:36	Stroke	None noted	None noted	16.99 ± 0.40[2]	3.66 ± 0.25
68	4:30	27:16	Lung cancer	No drusen noted. Variable choroidal thinning	No drusen. More choroidal thinning than fellow explant with loss of choroidal vessels	2.29 ± 0.0.30[2]	28.90 ± 0.23
68	3:22	44:27	Sepsis	Unknown (poor RPE preservation)	Unknown (poor RPE preservation)	6.19 ± 0.38[1]	19.54 ± 0.29
69	4:55	31:15	Renal failure	Small drusen	Small drusen	0.61 ± 0.08[1]	1.19 ± 0.10
69	3:54	30:20	Chronic obstructive pulmonary disease (COPD)	None noted	None noted	3.47 ± 0.30[2]	16.81 ± 0.39
70	5:03	47:40	Sepsis	Few small drusen	Few small drusen. Heavy BLinD	0[3]	0
71	2:30	42:00	Intra-abdominal abscess	No drusen noted. Heavy BLinD	No drusen noted. Heavy BLinD	0[1]	16.53 ± 0.25
71	4:40	28:10	Sepsis	Few small drusen	Few small drusen	0[2]	29.68 ± 0.33
73	5:31	46:31	Acute respiratory distress syndrome	Few small drusen, heavy BLinD	Few small drusen	0[2]	0
74	5:55	24:55	COPD	Heavy BLinD, forming superficial lumps. Choroidal thinning	Heavy BLinD forming superficial lumps, choroidal and cc degeneration in central macula	2.92 ± 0.43[1]	18.27 ± 0.49
74	3:45	48:55	Respiratory failure	None noted	None noted	2.25 ± 0.35[1]	15.12 ± 0.28
74	3:45	42:30	Metastatic lung cancer	None noted	None noted	8.55 ± 0.38[2]	15.18 ± 0.40

(continued)

Table 14.1 (continued)

Age	D:P	D:R	COD	Submacular pathology, hES-RPE explant	Submacular pathology, fRPE explant	hES-RPE ND ± SEM[a]	fRPE ND ± SEM
75	4:38	33:48	Renal failure	Several small drusen	Several small drusen	0[3]	8.02 ± 0.38
76	5:27	29:53	Septic shock	None noted	None noted	0[2]	7.26 ± 0.30
76	6:00	42:55	Intracerebral hemorrhage	Few small drusen	Few small drusen (less than fellow explant)	2.21 ± 0.25[2]	12.47 ± 0.49
77	5:17	43:00	Hypertension	None noted	None noted	10.36 ± 0.35[3]	12.70 ± 0.37
79	3:14	29:49	Pneumonia	None noted	None noted	0[1]	2.97 ± 0.22
79	4:30	47:00	Pneumonia	None noted	None noted	0[3]	18.32 ± 0.24
80	4:50	42:55	Cancer (type unknown)	None noted	No drusen noted. Severe degeneration of the cc and choroidal vessels	5.58 ± 0.25[1]	7.00 ± 0.50
81	5:30	42:30	Acute cardiac crisis	Large and small drusen	Unknown (poor RPE preservation)	0[1]	0
87	6:10	36:45	Cardiac arrest	Few small drusen, heavy BLinD	No drusen noted, heavy BLinD	0[3]	2.69 ± 0.41
93	4:05	46:55	Cardiac arrest	None noted	None noted	NA[b]	NA[b]

All donor eyes exhibited basal linear deposits (BLinD) extending into the inner collagenous layer of Bruch's membrane. Explants with substantial deposits are noted above

D to P death to preservation, *D to R* death to receipt, *COD* cause of death, *cc* choriocapillaris, *ND* nuclear density (nuclei/mm Bruch's membrane), *SEM* standard error of the mean

[a]Superscript after ND indicates the batch of hES-RPE (1, lightly pigmented, 2, moderately pigmented, 3 heavily pigmented)

[b]Data available for equatorial explants only (submacular explants contaminated). Reproduced with permission from Sugino et al. [22]

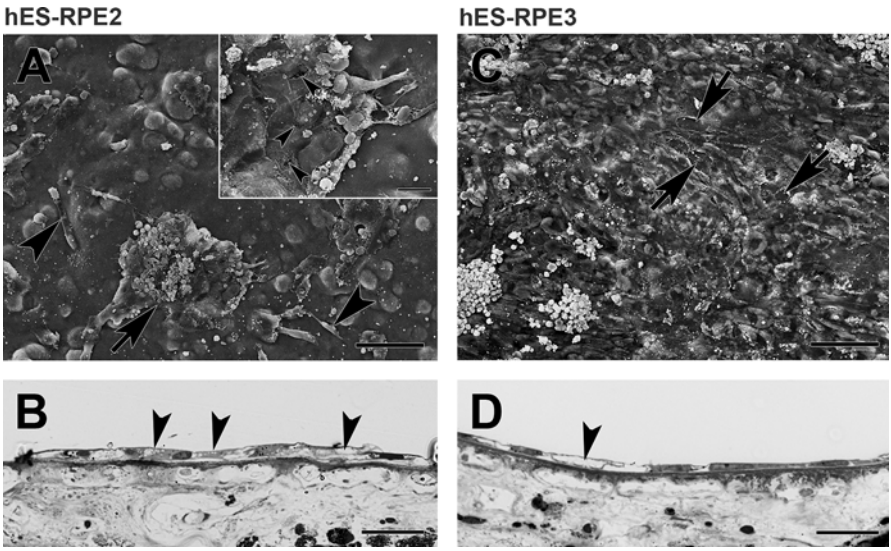

Fig. 14.2 Morphology of hES-RPE2 and hES-RPE3 on submacular Bruch's membrane after 21 days in culture (**a**, **b** donor age 69 years; **c**, **d** donor age 59 years). (**a**) hES-RPE2 show limited resurfacing of the explant by cell patches and elongated single cells (*arrowheads*). Rounded dead cells can be seen on top of the patch indicated by an *arrow* (ND, 3.47 ± 0.30). High magnification *inset* shows cells within the patch have membrane holes (*arrowheads*). Cells along the edge of the patch appear to be dead or dying. (**b**) LM of cells within a patch. Many of the cells contain vacuoles or show loss of cytoplasm (*arrowheads*). (**C**) hES-RPE3 have almost completely resurfaced the explant with cells that are highly variable in size and shape. Small defects in the coverage are indicated by *arrows*. Clusters of dead cells are present on top of the cell monolayer (ND, 12.84 ± 0.28). (**d**) LM of the explant shows resurfacing by elongated, flat cells, some with loss of cytoplasm (*arrowhead*). Scale bar: (**a**, **c**) 100 μm; (**a**, *inset*) 20 μm; (**b**, **d**) 30 μm. Toluidine blue staining. Reproduced with permission from Sugino et al. [22]

on BM may be quite important. Trophic factors secreted by transplanted cells may be an important component of their salutary effect on host retina [16, 26, 27]. Subretinal transplants of cells that are not RPE, for example, may rescue photoreceptors, at least in part, through neurotrophic factor secretion [26–29]. Neurotrophin secretion can occur even if the RPE are not fully differentiated. In the organ culture experiments described here, submacular BM explants exhibiting the best cell survival did not feature hES-RPE or fRPE with morphology of mature RPE. Thus, the encouraging visual results reported after transplantation of hES-RPE suspensions into patients with Stargardt disease and AMD may, in part, be a consequence of neurotrophic factor production by hES-RPE that may or may not be well differentiated but nonetheless survive on BM [30]. The limited survival of hES-RPE and fRPE on AMD explants, however, indicates that methods to improve cell survival on AMD BM may be important in advancing transplantation of RPE suspensions as a therapeutic approach.

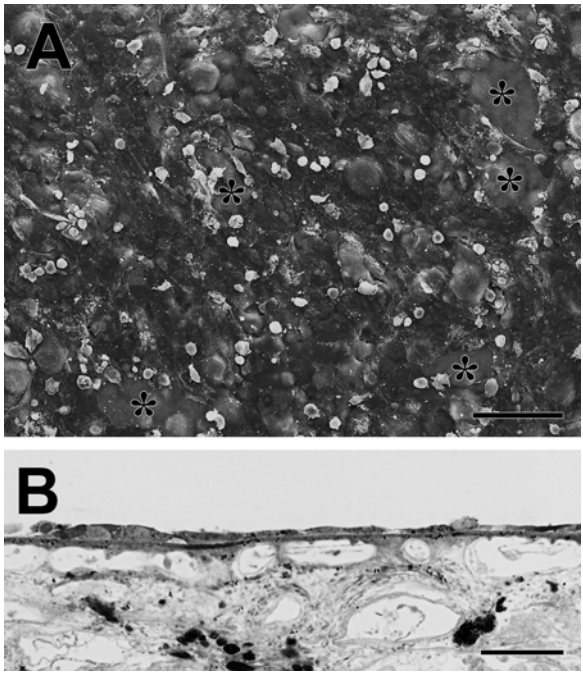

Fig. 14.3 Morphology of fRPE on submacular Bruch's membrane after 21 days in organ culture (donor age 69 years, same donor as Fig. 14.2a, b). (**a**) fRPE show more resurfacing of the explant than that observed by hES-RPE on the fellow explant. Cells on the incompletely resurfaced explant are very flat and highly variable in size. Large defects in cell coverage are indicated by *asterisks* (ND, 16.81 ± 0.39). (**b**) LM of the explant shows the variability in cellular morphology. Scale bar: (**a**) 100 **μ**m; (**b**) 30 **μ**m. Toluidine blue staining. Reproduced with permission from Sugino et al. [22]

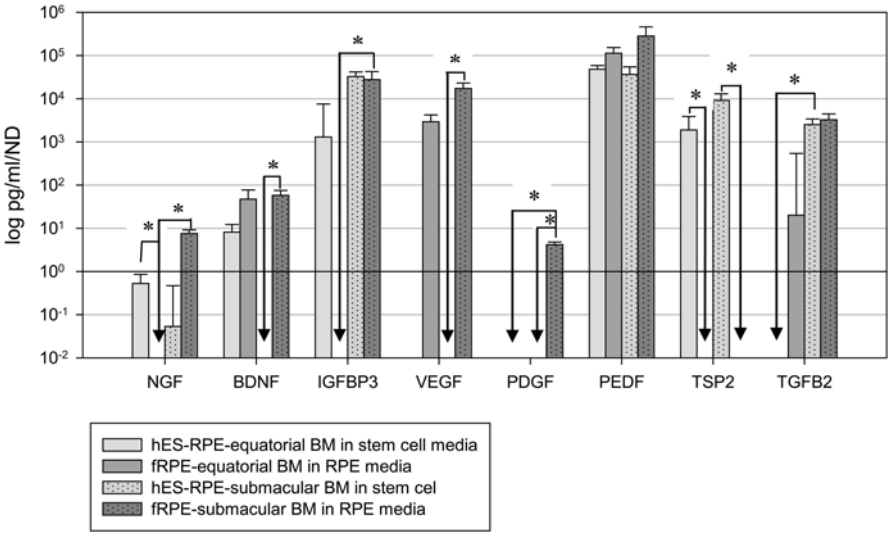

Fig. 14.4 hES-RPE and fRPE secretion on equatorial and submacular Bruch's membrane explants. Protein levels have been corrected for contribution from the explant and are normalized to nuclear density. Pairwise comparisons between explant preparations were performed for each protein. Significant differences between pairs are indicated (*$P < 0.05$). Reproduced with permission from Sugino et al. [22]

14.3 Enhancement of Embryonic Stem Cell-Derived RPE Survival on Human Bruch's Membrane

In contrast to results in humans with AMD [2, 3, 25], RPE transplants in animal models of retinal degeneration rescue photoreceptors and preserve visual acuity [18, 31–34]. An important difference between humans with AMD and laboratory animals in which RPE transplantation has been successful is the age- and AMD-related modifications of BM, which may have a significant effect on RPE graft survival [35]. Previous investigators used individual ECM ligands, singly or in combination, to improve RPE attachment to BM with limited success [36, 37]. In contrast, resurfacing human aged and AMD BM with bovine corneal endothelial cell (BCEC) ECM improved RPE survival in organ culture by more than 200 % [24]. BCEC-ECM is a biologically synthesized ECM that supports rapid RPE attachment, growth, and differentiation in cell culture [38]. BCEC-ECM contains ligands and growth factors present not only in appropriate amounts but also in proper three-dimensional array. We found, however, that BCEC-ECM did not have good surgical handling properties, and its relative insolubility rendered it difficult to analyze biochemically.

During BCEC-ECM formation, in addition to basal secretion, BCECs secrete ECM components into the overlying medium, including collagens, proteoglycans, and entactin/nidogen as well as proteases [39–42]. Secretion of ECM components into the overlying medium is most abundant in early passage cells and exceeds basal ECM deposition [39]. Soluble ECM can affect cell shape and metabolism and can stimulate the production of ECM molecules [43], so the presence of these proteins suggests that conditioned medium (CM) harvested from BCEC cultures (BCEC-CM) might improve RPE survival and differentiation on human aged/AMD BM and, if effective, could lead to the development of an adjunct to cell-based therapy for AMD.

Using the human BM organ culture paradigm, we found that BCEC-CM improved long-term survival of both hES-RPE and fRPE on aged and AMD BM by 400–1,000 % (Figs. 14.5, 14.6, and 14.7) [23]. The benefit of BCEC-CM was evident on aged BM, BM with geographic atrophy, and BM with choroidal new vessels from which the new vessels had been excised surgically (postmortem). Even adult RPE survival on BM was improved with BCEC-CM. ECM deposition was increased under the cells cultured in BCEC-CM compared with cells cultured in RPE medium. While increased ECM deposition may be a mechanism by which cell survival is enhanced (in the same manner that BCEC-ECM resurfaced explants support long-term RPE survival on submacular human BM), it is not clear that ECM deposition per se fostered cell survival or whether ECM deposition was a reflection of better long-term survival of the cells by another mechanism. Although the nuclear densities of hES-RPE and fRPE cultured in BCEC-CM were similar, hES-RPE tended to be flatter and somewhat less differentiated than fRPE cells on submacular BM. It may be that hES-RPE take longer to acquire mature RPE cell features on BM than fetal RPE, consistent with behavior observed in cell culture [15, 21]. To develop this modality further, we plan studies to identify the critical components of BCEC-CM and test RPE cell function following BCEC-CM treatment. Our goal is to develop a surgically usable adjunct to improve RPE cell survival and differentiation on submacular human AMD Bruch's membrane.

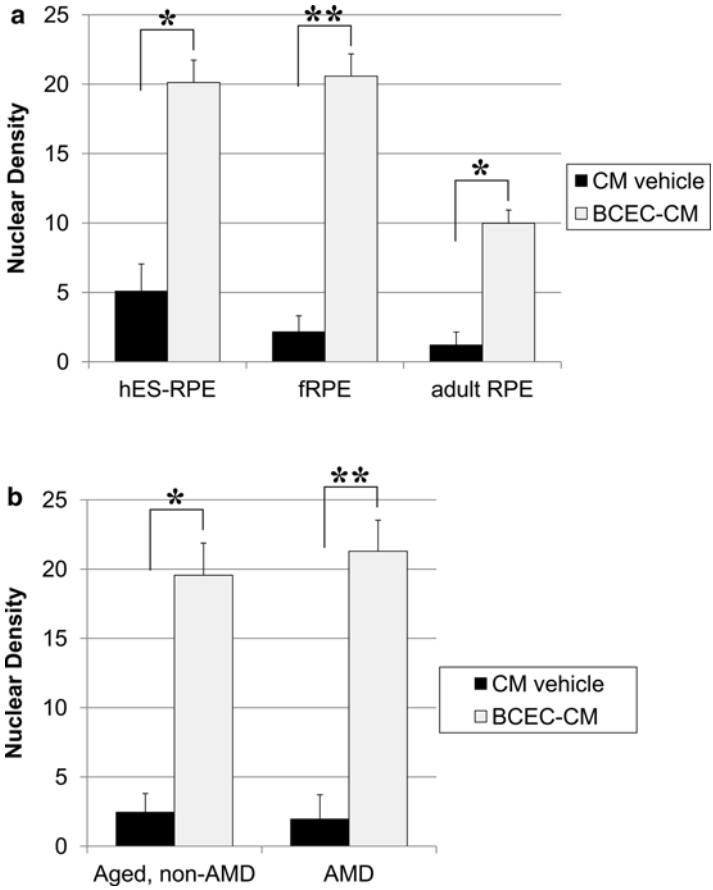

Fig. 14.5 Nuclear densities of cells seeded on aged submacular Bruch's membrane explants after 21-day culture in CM vehicle or BCEC-CM (paired explants from the same donor). (**a**) Nuclear density comparison of RPE cells derived from hES-RPE ($n=6$), cultured human fetal RPE (fRPE, $n=22$), and cultured human adult RPE (donor ages 58, 71, 78 years; $n=7$). Within each group, significant differences were observed between cells cultured in CM vehicle and cells cultured in BCEC-CM. The nuclear density of cells cultured in CM vehicle was not statistically different between groups. The nuclear densities of hES-RPE and fRPE were not significantly different from each other but were significantly higher than the nuclear density of adult RPE cells after culture in BCEC-CM. (**b**) Comparison of nuclear densities of fRPE on age-matched, non-AMD versus AMD Bruch's membrane at day 21. Explants seeded with fRPE on aged Bruch's membrane ($n=9$) were compared with explants seeded on AMD submacular Bruch's membrane ($n=13$). No significant differences were observed in the nuclear densities of fRPE on non-AMD versus AMD explants for a given medium, although the nuclear density was significantly higher in the presence of BCEC-CM versus CM vehicle. Nuclear density values are counts of nuclei of cells directly in contact with Bruch's membrane, expressed as mean nuclear density/mm Bruch's membrane. Bars indicate mean ± SE nuclear density. *$P<0.05$; **$P<0.001$. Reproduced with permission from Sugino et al. [23]

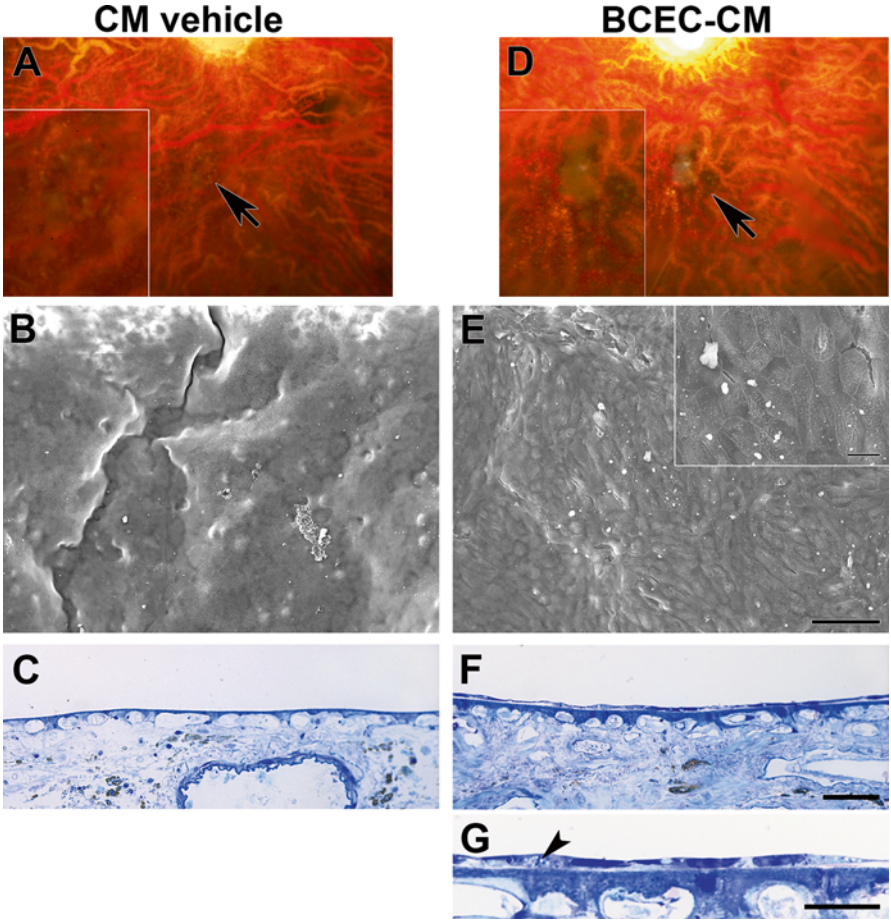

Fig. 14.6 Paired submacular explants from a 74-year-old woman with soft drusen, seeded with hES-RPE. In CM vehicle, (**a**) postmortem clinical photograph shows soft drusen (*arrow*) in the macula. *Inset* is a higher magnification image of the area indicated by the *arrow*. The drusen are not easily visualized in this photomicrograph because of postmortem changes. (**b**, **c**) No intact cells are seen on the cultured explant. In BCEC-CM, (**d**) *arrow* points to a patch of confluent soft drusen in the macula of the fellow eye, shown in the high-magnification *inset*. (**e**) Cells almost fully resurface the explant with small defects in coverage. Cells are variable in size and shape. (*Inset*) Cells are generally flat, with most exhibiting short processes on their surfaces. (**f**, **g**) Very flat, elongated cells resurface the explant in a monolayer. (**g**) *Arrowhead* points to cell-containing vesicles. CM vehicle nuclear density (ND), 0; BCEC-CM ND, 19.90 ± 0.35. Scale bars:100 μm (**e**); 20 μm (**e**, *inset*); 50 μm (**f**); 20 μm (**g**). Toluidine blue staining. Reproduced with permission from Sugino et al. [23]

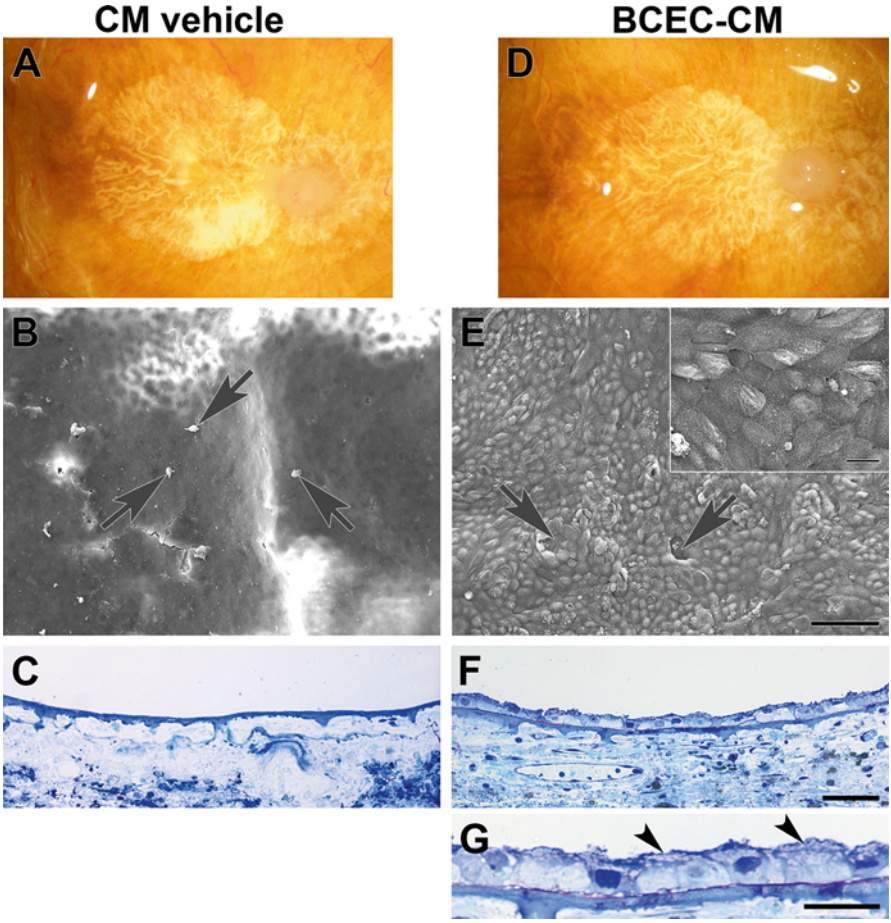

Fig. 14.7 Paired explants from an 82-year-old woman with geographic atrophy, seeded with fetal RPE cells. The patient's clinical history noted AMD for 20 years. (**a**, **d**) Postmortem clinical photographs showing subfoveal geographic atrophy before RPE cell seeding. In CM vehicle, (**b**) only a few dead cells (*arrows*) and cellular debris are present on the explant surface. (**c**) No cells are present on Bruch's membrane surface. In BCEC-CM, (**e**) RPE cells fully resurface Bruch's membrane in the area of geographic atrophy with a few very small defects (*arrows*). Localized areas of multilayering are present. Cell surfaces show abundant apical processes (*inset*). (**f**) In this field, cells resurfacing the BCEC-CM explant are predominantly bilayered. Cells directly on Bruch's membrane are small and tightly packed; flat cells appear to overlie the cells in contact with Bruch's membrane. (**g**) Flattened cell processes overlying cells on top of Bruch's membrane are indicated by *arrowheads*. The cell processes contain vesicles. CM vehicle ND, 0; BCEC-CM ND, 19.61 ± 0.43. Scale bars: 100 μm (**e**); 20 μm (**e**, *inset*); 50 μm (**f**); 20 μm (**g**). Toluidine blue staining. Reproduced with permission from Sugino et al. [23]

14.4 Conclusion

There are many strategic advantages to using stem cell-derived RPE for RPE replacement therapy. However, applications of cell-based therapy to AMD patients will require addressing the problem of long-term cell survival and differentiation on BM altered by age and AMD. One approach involves the use of scaffolds to shield the RPE from underlying BM [44–47]. Another approach, which we are exploring, is to use a soluble mixture of material to alter the extracellular environment favorably with the hypothesis that once the cells are in place for a sufficient period of time, they will elaborate their own ECM, which will shield them from the damaging signals present in AMD BM. Current efforts focus on identifying the bioactive components of BCEC-CM so that a humanized, clinically applicable product can be developed and deployed to improve the transplant success in patients. Finally, we note that the performance of MA09 cells used for these experiments could behave quite differently from the hESC-RPE used in clinical studies. The batches used for human transplants were thaw-formulated using new procedures that eliminate the majority of unhealthy cells, and only batches that passed postthaw criteria (establishing a uniform RPE monolayer, ≥95 % cells positive for RPE markers) were used. In addition, the cells used for human transplantation were derived and cultured using different procedures and media and have not been compared with those used for the Bruch's membrane organ culture experiments we report here. Thus, extrapolations from the preclinical data we report here to results in human transplants must be made with caution.

Acknowledgements Supported in part by the Lincy Foundation, the Foundation Fighting Blindness, an unrestricted grant from Research to Prevent Blindness, the Eye Institute of New Jersey, the Janice Mitchell Vassar and Ashby John Mitchell Fellowship, the Joseph J. and Marguerite DiSepio Retina Research Fund, the New Jersey Health Foundation, the Christopher Seery Retina Research Fund, and the New Jersey Lions Eye Research Foundation.

Commercial relationships. Ilene K. Sugino, U.S. patent applications, 20120210737 and 200100297234 (P); Qian Sun, none; Noounanong Cheewatrakoolpong, none, Marco A. Zarbin, U.S. patent applications, 20120210737 and 200100297234 (P).

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Chapter 15
Seeing the Full Picture: The Hidden Cost of the Stem Cell and Regenerative Medicine Revolution

Claire Tanner and Megan Munsie

Contents

Abbreviations

HIV	Human immunodeficiency virus
ISSCR	International Society for Stem Cell Research
IV	Intravenous therapy
SCT	Stem cell treatment
US	United States

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A. Pébay (ed.), *Regenerative Biology of the Eye*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4939-0787-8_15, © Springer Science+Business Media New York 2014

15.1 Introduction

In biomedical research communities it is somewhat of a truism to acknowledge the importance of investment in new and emerging biotechnologies. Stem cell science and regenerative medicine in this respect are no exception. In recent decades, significant time, money and energy have been invested in the attempt to harness the regenerative power of stem cells to ameliorate the pain and suffering in a wide range of illnesses and injuries—from autoimmune disorders, congenital diseases and degenerative neurological conditions to acquired brain and spinal cord injuries. For scientists and clinicians working in these fields, the idea of 'investment' is charged with particular meaning and is linked to pressures to access funding and the drive to provide assistance for people living with incurable conditions. Here stem cells are often posited as a 'holy grail' with magical powers just waiting to be unlocked or revealed to the world through the toil and labour of those pioneers at the cutting edge of research. It is in this heightened context of anticipation and expectation that significant investments are also made by those on the other side of the bench; the people and their loved ones seeking help for the conditions and illnesses with which they live and die. For some, this investment involves at least considering, and often travelling to receive, experimental stem cell treatments (SCTs). This chapter considers this costly and multifarious by-product of the stem cell and regenerative medicine revolution—the hope and resources invested by patients and carers in experimental SCTs.

Here we map some of the complexities for people who are faced with an often unreliable and conflictive congeries of information about stem cells and their treatment potential. To do so we draw on qualitative data from a pilot study undertaken in 2009–2010 into patient experiences of overseas SCTs entitled, *Hopeful Journeys*: *Experiences of Stem Cell Treatments Offered Outside Australia*, as well as preliminary findings from a current Australian Research Council-funded project entitled *High hopes*, *high risks*? *A sociological study of stem cell tourism*. The former involved 16 in-depth semi-structured interviews with patients and carers who had travelled overseas for SCTs [1]. The latter, interviews with stakeholders who provide information to people about SCTs ($n=20$); people and carers who have considered travelling for SCTs ($n=20$), and people and carers who have travelled overseas for SCTs ($n=20$) [2]. In the second study, the experience of those who are contemplating, or have had, SCTs in Australia are also being investigated. In all cases the SCTs that were being sought were not recognised as been established or 'proven' medical interventions.

We begin this chapter by considering community awareness and expectations around stem cells and the role of the media in presentations of stem cell science. We then address the growth of direct-to-consumer marketing of stem cells treatments more broadly, and for vision restoration in particular. Drawing on qualitative interviews with patients and carers who have travelled overseas for treatment, we then outline the journeys they undertake, including their motivations for travelling, perceptions of risk and benefit, and the significance of hope in their treatment decisions. We conclude by presenting a range of resources for health and medical professionals to draw on in their communications with people about SCTs. In so doing we offer a complex and contextualised picture for better understanding and

responding to the needs of the increasing numbers of people considering or travelling overseas or within their own country for experimental SCTs.

15.2 Community Awareness and Expectation in Stem Cell Research

In the popular imagination, the regenerative potential of stem cells is widely accepted. This is perhaps unsurprising given the prevalence of media reports which characteristically present stem cells as having extraordinary powers: to enable the blind to see, the paralysed to walk, the deaf to hear. Headlines in recent months alone include the following hyperbolic claims: 'Stem-cell transplants may purge HIV' [3]; 'Future blindness cure? Stem cell success in lab' [4]; 'Stem-cell treatment restores sight to blind man' [5]; 'Stem cell therapy helps 26-yr-old man walk again' [6]. The power of stem cells is further enhanced by the fact that SCTs have been used to successfully treat some diseases of the blood and immune system for over 50 years [7]. The necessary promotion of science and medical research, and the veracious capacity of the media to inflate and misrepresent advancements, has been identified as an ethical issue—and particularly in the fields of emerging biotechnologies—and is most often talked about in terms of a tricky 'balancing' act [8–11]. In particular, the ethical and policy issues associated with raising the profile of certain biotechnologies in order to respond to or attract public interest, and in turn investment in future research, have attracted critical attention (see e.g. [11–13]).

In the context of stem cell science, significant concerns have most recently been raised with respect to the relationship between the 'hype' around stem cells and the premature translation of this research into clinical settings. As Murdoch and Scott acknowledge, 'by rousing public excitement for the promise of stem cell technologies, stem cell supporters may have inadvertently contributed to the creation of a market for offshore treatment, enabling the very charlatans they now criticize' [14]. The exponential increase in providers in jurisdictions with little regulatory oversight who are marketing and selling unproven SCTs to local and overseas 'consumers' is therefore a key focus of concern; a phenomenon commonly referred to as 'stem cell tourism' [15–17]. A related deleterious consequence of the 'hype' surrounding stem cells also concerns community perception. Knowles [18], for example, has argued that inflated public perceptions about the status of SCTs prevent effective communication between patients and carers and health and medical professionals from whom they may seek advice about available treatments.

Our own research into patient understandings and experiences of SCTs also suggests that many people are heavily influenced by media reports, which often act as a catalyst for consideration of treatments not offered in their own country [19]. Here stem cells are both inadvertently and directly represented in the media, marketed by overseas providers and perceived by the public as a 'silver bullet'—a simple solution to a diverse range of complex and critical health and medical conditions. This is in part because many people have a positive and indiscriminate understanding of the function of stem cells to 'regenerate' and 'repair' the body, possibly drawn

by knowledge of conventional blood SCTs for leukaemia and similar blood disorders. The strength and generality of this belief means that it can apply to a broad spectrum of illnesses and conditions, as the following quotes from people with a range of conditions who travelled overseas for SCTs, indicate:

> And so we were hoping that, you know, these stem cells would help the areas of the brain that just weren't quite working to regenerate and to allow new pathways to be made. (Kate, mother of a child with significant developmental delays due to lesions on brain, recently travelled to China for umbilical cord stem cell treatment)
>
> The stem cells could go to the injury where your injury is, and basically grow to help repair I guess the, you know, the cord, to bridge the gap I guess, and that it could take time. They do, they do grow. (Pete, paraplegic, recently travelled to Germany for autologous SCTs)
>
> Um so yeah, look it was a case of ah stem cells …and stem cells only … get them to um, get to the site and help generate nerve endings…more nerve fibre. (Owen, father of a child with Optic Nerve Hypoplasia, recently travelled to China for allogenic SCTs)

As these quotes suggest, high expectations around stem cells' regenerative capacities mean that there is little delineation between the potential versus proven function of stem cells in repairing damage for a range of diseases and conditions. It is also possible that the strength of the association between stem cells and their regenerative potency means that perceptions of risk are comparatively low. This is in spite of the fact that there are very few conditions for which stem cells have been proven to be safe or effective [7]. Survey data indicates that in Australia, and similarly in Canada and the United States, community perceptions of the benefits of stem cell research are far greater than perceptions of risk [20]. In 2007 87 % of Australians believed that stem cell technology would have a positive impact and improve their way of life in the future [21]. In a similar survey conducted a couple of years later, 91 % of Australians considered 'using stem cells to conduct medical research and treat disease was useful' [22]. This survey data is particularly noteworthy as it indicates that stem cell science was perceived as having the lowest risk of any area of biotechnology.

So it is often with high expectations, and low levels of caution, that people and carers begin their search for relevant and helpful information. They must try to delineate fact from fiction in an online environment dominated by direct-to-consumer marketing of available treatments as a 'silver bullet', a subject to which we now turn.

15.3 Responding to Demand: The Growth of 'Stem Cell Clinics'

In recent years there has been an exponential growth in the number of websites targeting people hoping to procure better health through stem cells [23–25]. Many clinics offer treatments for a wide range of diseases and conditions, with providers rarely having recognised expertise and experience in relevant fields. These clinics claim to use adult autologous stem cells in their treatments—taken from the patient for their own use—as well as foetal stem cells, cord blood stem cells and embryonic stem cells. The mode of delivery of the stem cells also differs with stem cells commonly injected into the

body intravenously or by lumbar puncture [19]. Some clinics also advertise treatments involving injecting the cells directly into the brain or spinal cord [24].

Online marketers use a variety of techniques to compel potential customers to use their services including patient narratives, blogs, videos and links to scientific and news sources. Particular constructions of consumer and patient empowerment are mobilised by marketers who capitalize on the established promise of stem cells, whilst reassuring patients of the value and safety of treatments that are 'but a simple injection away' [25]. The power of emotive anecdotal evidence is instrumentally used to demonstrate the possible benefits of treatment, whilst emphasising patient effort, commitment and the prospect of slow yet significant improvement. The following excerpt taken from a website advertising treatments in China is worth quoting at length as it aptly captures this characteristic presentation of personal experience:

> Madison was diagnosed at four months old with septo optic dysplasia, along with the associated condition of optic nerve hypolasia. By clinical standards, she is completely blind. However, that is slowly changing. After several treatments, Madison saw a clock, but thought it was a button, and was able to see that a clothes hamper, a picture and a suitcase has been moved in her hospital room. "We were in shock," Dellinger said of seeing his daughter's vision improve for the first time. "It was a good feeling. Just knowing she even got that small of an improvement made it every bit worthwhile."
>
> Dellinger has seen Madison's reaction to the shiny, brightly-lit Christmas tree at his father's home. She never showed much reaction to the decorations in the past. "She just sits and stares at the Christmas tree at my dad's house," Dellinger said. "She is just in a daze with it and this is the first time she's ever seen it." But getting to the point of improvement was a tough journey. Madison's treatment consisted of four 30-minute intravenous (IV) therapy and four six-hour lumbar punctures over a month-long period of time. "The puncture is a small incision on the back and stem cells are injected," Dellinger said. "The difference is it goes straight to your brain through the spinal fluid as opposed to the bloodstream with the IV injections."
>
> Overall, Dellinger said his daughter did very well with the treatment. Doctors expect the treatment to take effect in a year, but Dellinger has already seen improvement. [26]

The increase in direct-to-consumer marketing of unproven SCTs has prompted interventions from organisations providing support for patient/carer networks, as well as various national and international scientific organisations concerned about patient welfare, including the International Society for Stem Cell Research (ISSCR). In 2008 ISSCR emphasised the pressing need to address the proliferation of clinics directly marketing unproven SCTs to people with critical health needs, stating:

> Numerous clinics around the world are exploiting patients' hopes by purporting to offer new and effective stem cell therapies for seriously ill patients, typically for large sums of money and without credible scientific rationale, transparency, oversight, or patient protections. The ISSCR is deeply concerned about the potential physical, psychological, and financial harm to patients who pursue unproven stem cell-based 'therapies' and the general lack of scientific transparency and professional accountability of those engaged in these activities. [27]

Recent findings from a comparative review of online advertising of stem cells in 2008 and 2013 indicate that despite these kinds of interventions and the increased scrutiny of stem cell tourism more generally, there has been little impact on the kinds of claims clinics make about the treatments they offer in their online

advertising [28]. Importantly, whilst the majority of these clinics operate in jurisdictions with comparatively lax regulatory guidelines, for example in India, Malaysia and Thailand (for the latter see [29]), increasing numbers of 'backyard' providers are operating in countries that are understood to be highly regulated (for the US see [30]). Australia offers a particular example.

Over the last three years, there has been a dramatic increase in the number of doctors and clinics in Australia offering autologous SCTs. Under the current regulations, such treatments do not fall under the stringent requirements set by the Australian regulators, the Therapeutic Goods Administration, provided the treatments are administered to individual patients by a registered Australian medical practitioner [31]. None of the treatments on offer are considered 'standard medical practice' or have been subject to peer review. Most, if not all, are conducted outside the context of a clinical trial with the cells rarely prepared in laboratories adhering to Good Manufacturing Practice. Despite these failings, the treatments are being marketed, often for a considerable fee, for a wide range of conditions (such as stroke, Multiple Sclerosis, Motor Neurone Disease, autism and osteoarthritis). While simple modifications to the current regulations—such as incorporating recognition of the inherent risks in extending the use of cells beyond what they usually do in the body (i.e. non-homologous use) and making it a requirement that cells are prepared in accredited laboratories—could curb these practices, many Australian scientists and clinicians fear that these unproven SCTs will continue to be available until reports of significant complications trigger professional misconduct investigations and a change in the regulations [31].

The marketing of unproven autologous SCTs, and a lack of regulatory response, is not just an issue for Australia. Concerned about the proliferation of these practices across the globe, the ISSCR recently released a statement calling for regulators, patient advocacy organisations, doctors and others to discourage the sale of unproven treatments using the patient's own stem cells outside clinical trials, going so far as to call such practices unprofessional and unethical [32].

Beyond the financial costs, the risk of physical harm from unproven SCTs—no matter the source of cells—is real. Although thankfully rare, reported complications include infections and tumour formation [33–36], and even death [37, 38]. Such possible risks to future health, or the inherent risk of the intervention itself, are seldom acknowledged by providers. It is perhaps unsurprising then that many patients only view risk in terms of financial loss, as Lisa commented,

> It came down to the worst that could happen was nothing really, the worst that could happen was we could spend our money and it could have been, we could have gotten no result. (Lisa, mother of a child with Cerebral Palsy who recently travelled to Germany for autologous SCTs)

We have also observed that perceptions of physical risk, when acknowledged, are the lowest when people understand their own cells to be used for treatment. Such impressions are reinforced by online advertisements within Australia and overseas which promote autologous SCTs as having 'no risk' as they are 'natural'. The recent report of bone fragments growing around a patient's eye following a stem cell 'facelift' highlights the potential hazards of early adoption of unproven SCTs [39]. Other studies also indicate that the safety of autologous stem cells has not been adequately

established [40–42]. In one case, multiple lesions developed at the site of autologous SCT injections [35].

In spite of calls for extreme caution and concern in light of these perceived risks, many people and carers are travelling to overseas destinations in the hope for some improvements in their conditions. The following sections, drawn from our research, provide some further insight into the experiences and understandings of those who have embarked on these journeys, as well as the challenges and experiences of those stakeholders who provide information to people considering travelling for SCTs.

15.4 The Patient Experience: Hopeful Journeys

In the absence of established scientific evidence, patients and carers considering SCTs highly value communication with people who have already undergone treatment. It is common for providers to facilitate patient-to-potential patient communication, for people to use the Internet to source contact details for those who have told their story to the media and for people to communicate online through blogs and patients forums, as Emily's account below indicates:

> After reading some of the patient blogs, like you didn't get a lot of information so I actually contacted one of the families in America because that was a success story. […] So I contacted them and basically asked them what was involved and that sort of thing and that was when we decided, yes it's worth going to China for the treatment. (Emily, mother of a child with Optic Nerve Atrophy, recently travelled to China for SCTs with donated cord blood)

People and carers often consider evidence of this kind the most helpful in deciding whether or not to travel, and the best indicator of potential outcomes and effectiveness. Many are also defensive about personal accounts of success and improved function being pejoratively characterised, as David's comment captures:

> Interviewer: So, in terms of your evidence that I guess justified that leap of faith, as you said, it was based on that kind of legitimacy around the clinic, and that anecdotal evidence from people who'd had the treatment and, [Yep] that it was a -
>
> Well not, not anecdotal. I mean the fact of the matter is there was a guy with three broken vertebrae who is now running in half marathons. It's not anecdotal; that's fact. That's actual. (David, spinal cord injury, travelled twice to Germany for autologous SCTs)

Importantly however, people do not necessarily have high expectations of treatment but are motivated to undertake treatment due to the lack of options in their home countries. As Kylie describes:

> I actually, sort of probably sounds a bit bizarre, but I really went into the treatment not expecting anything, so that any benefits I received was a bonus, you know, I was totally aware that I might not get any benefits but because I had no other, no other opportunities for treatment, I thought well I'll give stem cells a go. (Kylie, travelled twice to China for allogenic SCTs for Multiple Sclerosis)

As already indicated, people's perceptions of risk were also low, with financial risk being universally identified as the greatest concern amongst participants. For many, the costs of treatment—which ranged from $6000 to 60,000 plus accommodation

and airfares—were extremely prohibitive and were an obstacle that had to be overcome. How people met the costs varied. It was common for people to have undertaken often extensive fund-raising campaigns in their local communities, mortgaged their house or spent their own or their parents' superannuation. For many, like Emily, this financial burden was exacerbated by a sense of urgency to access treatment in order to maximise any possible benefits. For many people 'waiting' for alternative treatments was therefore not an option:

> I can't afford to sit around and wait because with [our child] being totally blind the longer we wait the more retraining he's got to do if he does get any sight at all. (Emily, mother of a child with Optic Nerve Atrophy, recently travelled to China for SCTs with donated cord blood)

Significant financial investment occurred alongside other forms of investment. In people's accounts, hope played profound and complex roles in the decisions to undergo treatment, experiences of treatment and reflections on having had treatment. In meeting the day-to-day challenges of critical illness and disability, whether people had travelled for SCTs or not, hope was a motivator, coping strategy, reassurance, catalyst for action and a provider of meaningful purpose:

> Well [hope] it's a big thing. Even now like you've still got to hope that stem cells in five or 10 years are going to do something. I may never fully walk again but I might be able to stand or go to the toilet normal, or something like that. So yeah, yeah, definitely hope is, if you haven't got hope, you're going to struggle through things, that's for sure. It keeps you motivated to go and train, and get up in the morning, and go to work, and all the rest of it. (Pete, paraplegic, travelled to Germany twice for autologous stem cell treatment)
>
> I think without hope there's nothing. You know, if you don't have hope, I don't know how you keep going with something like this. (Gemma, carer of her husband with form of muscular dystrophy, considering SCTs)
>
> Richard: Well I mean without hope, I mean at the moment you just accept and feel there is no hope, and you stop. I mean then basically you're stopping any chance for your child to improve. So hope, without hope I mean … Even, even if it's false hope, even if practitioners, even if really there isn't any chance having, having hope allows for -
>
> Sadie: For us to give more love to our child. For him to grow up to be a happier, more together person and just for our family environment, it allows us to sort of love and appreciate him so much more, and, and we feel like each unit, each one of us in the family is really, really precious and sort of Charlie is just our centre and focus. (Husband and wife with a child with Cerebral Palsy, travelled to Germany and China for SCTs with donated cord blood)

The power of hope for patients has long been recognised in other fields of medical research and treatment, most notably in oncology [43–45]. As the comments above indicate, in the context of stem cell research, hope is a critical and multi-faceted resource for people in sustaining and supporting relationships, and people's ability to function day-to-day. In this respect SCTs thus offered for many people significant benefits beyond clinical improvements to their condition [19]. As one participant explained,

> I have not regretted it for a day. It certainly made a difference and as I said, it's given me hope. (Natalie, Spinal Injury, travelled to India for allogenic SCTs)

Like Nathalie, participants in our study almost universally reflect positively on having travelled for SCTs irrespective of clinical outcomes. Most described minor improvements in function, as well as other benefits including and beyond physical improvements, which were of great significance to them.

The challenge of managing hope and offering clear and accurate advice and information in responding to the issue of SCTs is a daunting task. The final section of this chapter considers some of the resources and important considerations for those medical and health professionals faced with the often confronting and difficult task of providing information to people considering SCTs as a possible treatment option and being sensitive to their powerful investment in SCTs as a source of hope.

15.5 Managing Expectation in Stem Cell Science and Regenerative Medicine

In response to what is seen as misinformation being promulgated by the providers of unproven SCTs, and echoed by enthusiastic media, many eminent national and international organisations have produced resources, in many different languages, to raise awareness about this issue (see Table 15.1). These resources provide pertinent information about current research into stem cells and are designed to highlight the need for clinical trials to properly evaluate possible new treatments, whilst raising awareness of the paucity of scientific evidence supporting unproven SCTs and possible implications of proceeding. The resources raise issues such as financial risks, physical harms, unlikeliness and uncertainty around possible benefits; deviation from more established modes of care and possible ineligibility for participation in future clinical trials. Many of the resources also include helpful questions that the patient should ask of any provider and encourage those investigating possible SCTs to discuss the findings from their research with a doctor who is not directly involved in treatment they are contemplating.

Managing the expectation of people looking to stem cells and regenerative medicine to restore vision, or enable vision in those who have not been born with this ability, in particular raises additional issues. Although blindness may result from damage to the outer surface of the cornea in an industrial accident, congenital disorder or degeneration of the retina or optic nerve as a result of illness or aging, many of the clinics offering SCTs do not distinguish between such causes. Rather they offer, as described earlier, a ‘silver bullet’ capable of seeking out the site of damage or dysfunction and restoring function. This belief is further compounded by media reports of early success from clinical trials using stem cells [46]. While the increasing number of clinical trials now underway using stem cells for conditions such as age-related macular degeneration, Stargardt’s disease, retinitis pigmentosa and corneal repair [47, 48], are very encouraging, for those who want treatments now and may not have access or be eligible for a clinical trial, available unproven SCTs are even more alluring. This example highlights that while the resources cited in Table 15.1 are valuable, more condition-specific information needs to be developed to contextualise the science and the progress being made for a particular disorder to counter the ‘one size fits all’ model of most SCT providers.

Although making more information available will be of some benefit, how the information is delivered and what else is said are important considerations.

Table 15.1 Resources for patients and doctors wanting to find out more about stem cell therapies and how to distinguish established clinical use from providers of unproven treatments

Resource	Organisation	Link
Patient Handbook on Stem Cell Therapies[a]	ISSCR	www.isscr.org/home/publications/patient-handbook
The Australian Stem Cell Handbook	National Stem Cell Foundation of Australia and Stem Cells Australia	www.stemcellfoundation.net.au/patient-information/handbook
What you need to know about stem cell therapies	Canadian Stem Cell Network, Health Law Institute and Albany Medical College	http://www.stemcellnetwork.ca/index.php?page=patientbooklet&hl=eng
Stem Cell Treatments—A Quick Guide for Medical Practitioners	National Health and Medical Research Council—Australia	http://www.nhmrc.gov.au/guidelines/publications/rm001
Stem Cell Treatments—Frequently Asked Questions	National Health and Medical Research Council—Australia	http://www.nhmrc.gov.au/guidelines/publications/rm001
A Closer Look at Stem Cell Treatments website	ISSCR	www.closerlookatstemcells.org
What are stem cells? website[a]	Knoepfler Lab Stem Cell Blog	www.ipscell.com/scope-global-stem-cell-outreach-program-for-education/
For Patients website[b]	Canadian Stem Cell Network	www.stemcellnetwork.ca/index.php?page=for-patients&hl=eng
What diseases and conditions can be treated with stem cells? website	EuroStemCell	http://www.eurostemcell.org/faq/what-diseases-and-conditions-can-be-treated-stem-cells
Stem cell fact sheets[b]	EuroStemCell	http://www.eurostemcell.org/stem-cell-factsheets

[a]Available in multiple languages
[b]Includes links to condition-specific information

Our research indicates that many people express considerable frustration and disappointment about how medical professionals and scientists respond to them when they raised possible SCTs or discussed their experience of SCTs upon their return. As the following comments reflect:

> I was very disappointed …he didn't want to talk to me and then when he eventually did talk to me there was absolutely nothing gained, so I guess I felt I was probably disappointed to think at the time that I didn't know where I could go for any information here in Australia so I thought well, "I'll give it a shot". (Victor went to India twice for allogenic SCTs for his spinal cord injury)
>
> But, when he said that [SCTs don't work], I was sort of quite devastated because there was this hope and it was being, you know, "Don't, don't take that hope away from me." (Jill, husband diagnosed with a form of dementia 12 weeks prior to interview, considering SCTs)
>
> Oh doctors … they just really don't want to discuss it. They just think that you're never going to get better and there's something wrong with you because supposedly you can't accept the situation and you're exploring potential opportunities but they don't think that's worthwhile doing. (Alex, Spinal Cord Injury, considered SCTs)

Such dismissive responses alienated people in need. For those at the start of their research, such as Victor, an inability to engage with their treating doctor acted as a catalyst for their independent online enquiry. Others indicated that similar responses made them reluctant to further discuss SCTs with Australian medical professionals.

For parents seeking treatments for their children, there are even further complexities [23]. With the perception that there may only be a narrow developmental window in which the SCTs will be beneficial, parents face unique pressures. As Harry explained:

> We were just happy to get on and do it and while [our daughter] was young as well. Got the greatest chance of making a difference while she's still young and at such a stage of development. (Harry father of a child with Cerebral Palsy who travelled to Germany for autologous SCTs)

The rights of the child and the potential conflict of parental consent must be acknowledged. As Reimer et al. rightly point out, 'parents, physicians, and policymakers must not lose sight of the harm that exists in excluding children and adolescents from decision making and self-determination' and that 'a critical step' in educating parents about stem cell tourism also involves 'encouraging and engaging in age-appropriate communication with young people' about risks and benefits of SCT [49].

What is required when approached by people contemplating experimental SCTs is open-minded, sensitive and clear communication involving 'more than providing decision makers with the right information' [50]. Models of best practice now and in the future will arguably take careful and considered account of the experiences, understandings and values of those seeking SCTs. This needs to include acknowledgement of other outcomes. For health and medical professionals, acknowledging this important aspect of patient experience is key to maintaining open and constructive dialogue with those seeking information for available treatments. For those charged with the responsibility of responding to enquiries about experimental SCTs this also means recognising and valuing the profound role of 'hope' for people in the management of their or their loved ones' conditions [19, 50]. By reframing the discussion with patients and their carers, taking their values and experiences—especially their hope—seriously, only then may we be able to better

assist and reduce the great cost for those deeply invested in the promise of stem cells and the regenerative medicine revolution.

Acknowledgments We would like to acknowledge our colleagues Prof. Alan Petersen and Dr. Kate Seear for their valuable contribution to the research that informed this chapter. We are also very grateful for the support of the patient/carer support networks who helped us recruit participants for our studies. Finally, and most importantly, this work would not be without the people who shared, with generosity and candour, their personal journeys to find a better quality of life. To each of the participants, thank you.

This research was funded by the Australian Research Council under the Discovery Projects scheme (DP120100921), and the Australian Government's Department of Innovation, Industry, Science and Research under the National Enabling Technologies Strategy (NETS). Ethics clearance was obtained from the Monash University Human Research Ethics Committee (CF12/1532–2012000832; CF08/3073–2008001514; CF09/3234–2009001762). Pseudonyms were assigned to protect participants' anonymity. Stem Cells Australia is a Special Research Initiative in Stem Cell Science funded by the Australian Research Council.

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DOI 10.1007/978-1-4939-0787-8,