The RPE in Myopia Development

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Yan Zhang and Christine F. Wildsoet

Introduction

Myopia, or near-sightedness, is one of the most common types of refractive error, which are major causes of visual impairment and blindness worldwide [1–3]. Refractive errors occur when there is a mismatch between the refractive state or focusing power of the eye and its axial length. They reflect the product of complex developmental interactions between various ocular tissues and compartments, including the cornea and crystalline lens, which together provide refracting power, and the anterior and vitreous chambers, which are the major determinants of overall eye length [4, 5]. Myopia describes the case when the eye is relatively too long and the image plane for distant objects falls in front of the retina. Most myopia is axial rather than refractive in nature and the product of excessive elongation of the vitreous chamber [5, 6]. While the problem of blurred distance vision resulting from such focusing errors can be corrected, for example, with optical appliances, the excessive eye growth carries an increased risk of a number of ocular pathologies, including cataract, retinal detachment, myopic maculopathy, glaucoma and choroidal neovascularization, many of which can lead to irreversible vision loss, with no evidence of a safe threshold level of myopia [7, 8].

In recent years, both the prevalence and severity of myopia have increased rapidly worldwide, albeit with variations between regions and ethnic groups [9–12]. While the estimated global prevalence of myopia was only 28% in 2010, it is now projected to increase to 50% by 2050 [10]. In some subpopulations of East Asia, the reported prevalence of myopia has already reached 80–95% [9, 13, 14]. While there has also been a tendency to view such prevalence figures as an Asian problem, Western countries are not exempt from this myopia epidemic [12, 15]. For example, one United States-based study reported an increase in the prevalence of myopia from 25 to 42% over a 30-year period [12]. The global prevalence of high myopia is also increasing, with a projected figure of 10% by 2050, up from 4% in 2010 [10]. As the risk of secondary ocular complications increases with the level of myopia, the latter figures represent a major public health concern. Indeed, myopia is now recognized as a significant public health problem, both socially and economically [2, 13, 16, 17].

What is the origin of the myopia epidemic? It is generally accepted that myopia is likely a product of gene-environment interactions, rather than being determined by either genetic or environmental factors alone [18–22]. Genetic contributions to myopia have been investigated for both familial non-syndromic (simple) and syndromic

Y. Zhang · C. F. Wildsoet (✉)
School of Optometry, University of California, Berkeley, CA, USA
e-mail: wildsoet@berkeley.edu

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forms. Linkage studies have mapped two dozen loci and identified mutations in a few genes. More recently, genome-wide association studies (GWAS) have been used to screen for myopia candidate genes. Many of the identified candidate genes have been linked to biological processes, with plausible connections to retina-sclera signaling cascades and thus local eye growth modulation. Examples include retinal neurotransmission, ion transport, extracellular matrix (ECM) and connective tissue remodeling [23–25]. Nonetheless, variants identified in GWAS studies explain only ~3% of the variation in myopia prevalence [26]. On the other hand, human epidemiological studies have provided convincing evidence for influences of environmental factors, such as excessive near work and outdoor activities in myopia development and prevention, respectively [27–30]. However, contributing factors are poorly understood and it is likely that interactions between genes and environmental factors at least partly explain the apparent complexities associated with predicting human myopia.

Despite the now general acceptance of the seriousness of the climbing myopia prevalence statistics from a public health perspective, treatments for preventing myopia and/or slowing its progression and thus controlling this epidemic remain limited in both options and overall efficacy [31]. Currently, therapeutic interventions are confined to methods of slowing myopia progression and thus limiting the level of myopia and risk of pathology [32]. While standard optical appliances (spectacles and contact lenses) and refractive surgeries are able to restore sharp distance vision, they have no positive benefits on myopia progression. Nonetheless, some specialized spectacles and contact lenses with multifocal optics have been shown to slow myopia progression [33, 34], and another contact lens modality, orthokeratology, which induces multifocal ocular changes after overnight wear, has also been shown to slow myopia progression [35–37]. In terms of drug therapies for controlling myopia progression, trials of topical ophthalmic medications have been limited to two muscarinic receptor antagonists, atropine and pirenzepine, both of which have been shown to slow myopia progression [38–42]. However, the underlying mechanisms for their therapeutic actions, including their site of action, remain the subject of debate. The same is true for a third drug, 7-methylxanthine, which has been approved for use as an oral medication in Denmark [43]. Among reported limitations and side-effects of the above treatments are ocular infections, due to poor handling of contact lenses and corneal changes with orthokeratology, as well as loss of efficacy and rebound effects with more traditional, higher concentrations of topical atropine [31]. Apart from the more conventional optical and pharmacological interventions, the results of epidemiological studies showing an apparent protective effect of outdoor activities against myopia onset and to a less extent, against progression, await a better understanding of contributing factors for their translation into behavioral recommendations [27, 29, 30].

RPE Changes in Human High Myopia and Related Pathology

Although few pathological changes have been reported in patients with mild to moderate myopia, these changes increase significantly with high myopia (generally defined as <−5 or −6 diopters [D]) [44–46]. Since the major ocular change in myopia is an enlargement of the posterior vitreous chamber, it is not surprising that pathological changes in high myopia are also largely limited to posterior ocular tissues, including retina, retinal pigment epithelium (RPE), Bruch's membrane, choroid and sclera. In myopic maculopathy, RPE changes, including RPE atrophy or loss, are among the frequently described features contributing to the characteristic fundus appearance [45]. Lacquer cracks, another commonly encountered fundus feature of high myopia, are believed to be caused by mechanical linear breaks in Bruch's membrane, and in patchy chorioretinal atrophy, holes in Bruch's membrane have been detected with new imaging technologies, such as swept-source optical coherence tomography

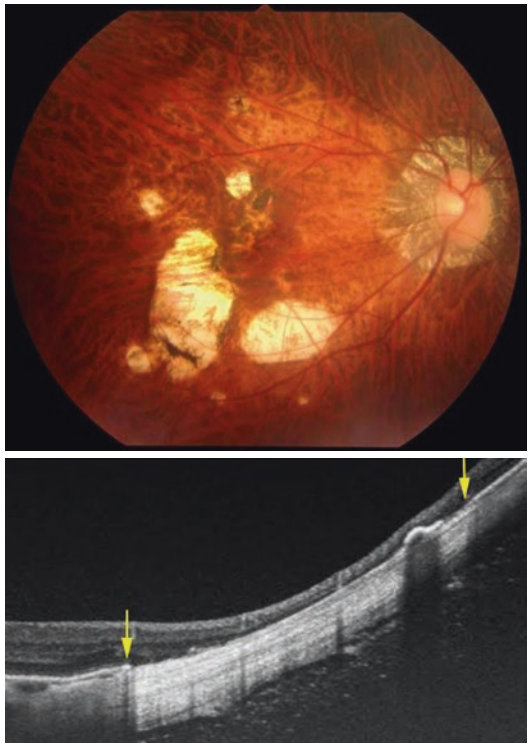

Fig. 7.1 Fundus photograph from a myopia patient with chorioretinal atrophy showing multiple patchy lesions. An OCT image from the same patient shows a RPE defect in the area of atrophy (between arrows). (Images courtesy of Professor Kyoko Ohno-Matsui (Tokyo Medical and Dental University, Tokyo, Japan))

(SS-OCT) (Fig. 7.1) [45, 47]. Such findings are also consistent with observations from more classical, histological studies of highly myopic eyes, of RPE losses and Bruch's membrane defects [48].

Role of RPE in Eye Growth Regulation and Myopia: Experimental Studies

Local Regulation of Eye Growth and RPE as a Relay Station

Although changes in the RPE are commonly observed in high myopia, such changes are usually considered to be secondary to the stretching of posterior ocular tissues as the vitreous chamber enlarges. The active contributions of RPE to later complications, as well as myopia onset and early progression are not very well understood.

Research studies on key ocular tissues such as retina, RPE, choroid, and sclera from myopic patients have been limited by the availability of tissue [49–51]. As substitutes, researchers have relied on a number of different animal models for myopia, including chicks, guinea pigs, tree shrews, marmosets and monkeys, with mice and zebrafish having been also featured in some more recent studies [19, 52–59]. Compared to the more widely studied earlier models, the latter models offer advantages of well-characterized genomes, as well as the availability of genetically modified animals.

Studies using animal models have provided convincing evidence for the role of visual environmental factors in eye growth regulation and myopia development. Most animals exhibit refractive errors at birth and, under normal conditions, these neonatal refractive errors are eliminated through a process of active, vision-dependent emmetropization, which involves the coordinated growth of key ocular components. Evidence for the latter comes from studies involving manipulations of the visual experience of young animals experimentally, using either optical defocusing lenses or form depriving diffusers. All of these disrupt this emmetropization process, with refractive errors being the net result. Specifically, hyperopic optical defocus (imposed with negative lenses) and form-deprivation (wearing diffusers) represent robust methods for stimulating excessive eye elongation, inducing myopia, while myopic defocus (imposed with positive lenses) slows eye elongation in most models. These experimental paradigms have been capitalized on in animal model studies aimed at understanding underlying mechanisms. Two lines of evidence point to these altered eye growth patterns being largely controlled locally, within the eye itself (Fig. 7.2) [19, 60–67]. Neural lesioning studies involving optic nerve section offer the strongest evidence for local ocular control; specifically, while the retina-brain link is thus disrupted, the usual response to negative lenses and diffusers, of increased eye elongation and myopia, is not [63, 65, 67]. Additional evidence comes from studies involv-

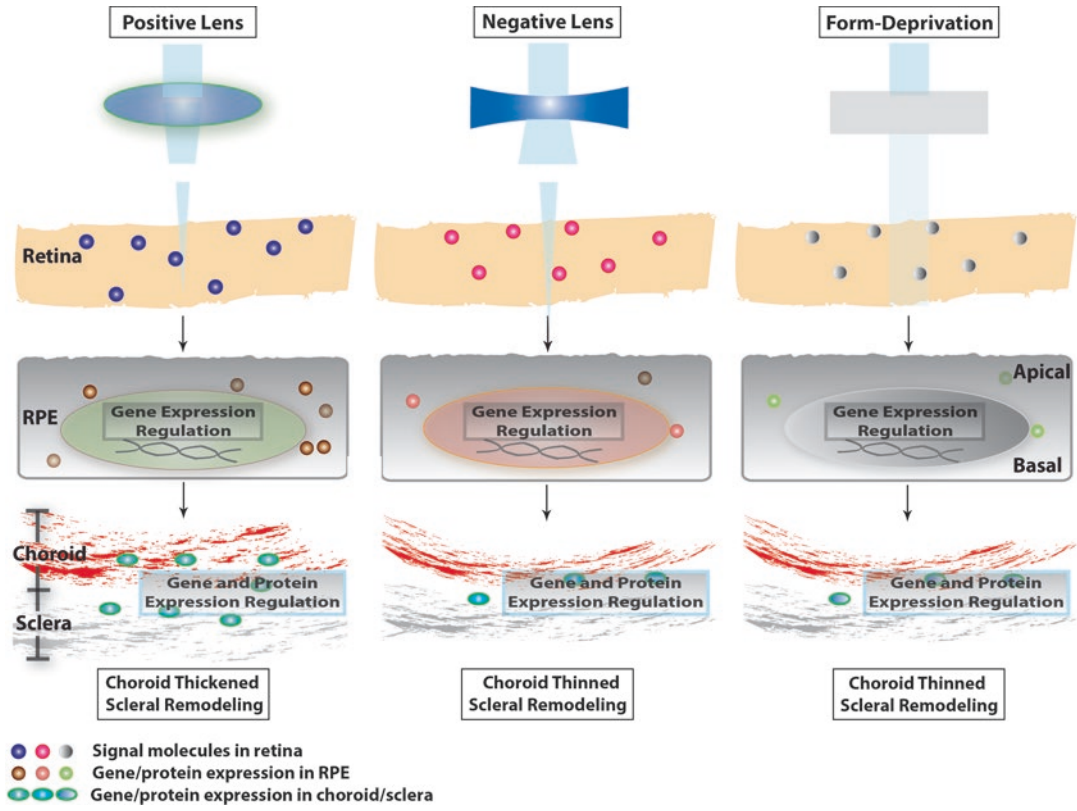

Fig. 7.2 Schematic diagram illustrating the presumed retina-sclera growth modulatory signaling cascades mediating positive lens-induced hyperopia, negative lens-induced myopia, and form-deprivation-induced myopia

ing localized manipulation of retinal images; in these cases, ocular growth changes are confined to the affected segment of the vitreous chamber [60, 64, 68]. The most parsimonious explanation for these observations relies on the assumption that the neural retina is linked via one or more local molecular signal cascades, directed at and mediating the morphological changes in the two outer layers of the eye wall—the choroid and sclera, which ultimately determine the physical dimensions of the vitreous chamber and thus eye length. By manipulating the retinal image and thus neural activity within the retina, the growth modulating signals are altered, with refractive errors being the net result (Fig. 7.2) [19, 69–73].

The RPE is a monolayer of hexagonal-shape pigmented epithelial cells positioned between the neural retina and the choroid (Fig. 7.3). Interconnected by tight junctions, the RPE cells form a key component of the blood-retina barrier, which tightly regulates the exchange between the retina and choroid of ions and water, as well as many molecules, including nutrients, and waste products (Fig. 7.4). The polarized nature of the RPE necessitates orientation labeling; the so-called apical membrane refers to the microvillus surface into which photoreceptor outer segments project, while the basal membrane refers to the surface facing the choroid and abutting Bruch's membrane. Many of the functions of RPE exhibit directionality, including the secretion of growth factors and cytokines, and there are also asymmetries in the distributions of neurotransmitter receptors, ion channels and transporters across the apical versus basal membranes [74–78]. Due to its unique location, juxtaposed between the retina and choroid, and barrier function, the RPE not only plays essential roles in maintaining retinal homeostasis and functions, but also plays important roles in maintaining choroidal

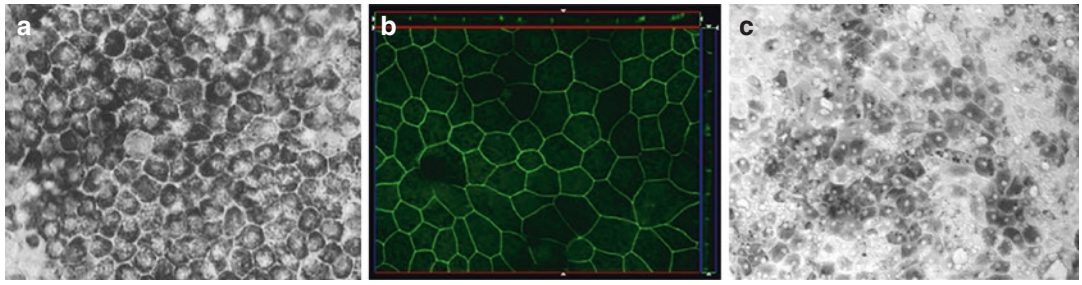

Fig. 7.3 Primary culture of human fetal RPE cells (**a**, **b**) and chick RPE cells (**c**). Confluent primary human fetal RPE cells in culture form tight junctions, as revealed with immunohistochemistry (ZO-1 labelling, **b**)

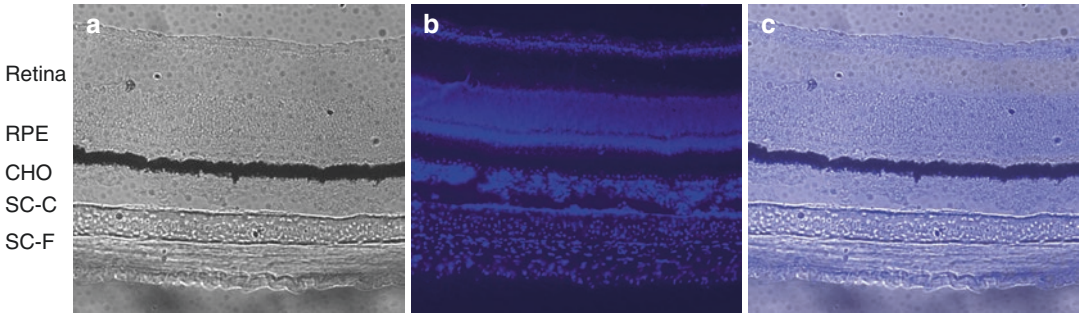

Fig. 7.4 Histology sections of the posterior wall of the chick eye, showing retina, RPE, choroid (CHO), and bilayered sclera (cartilage layer, SC-C; fibrous layer, SC-F) viewed under light microscopy (**a**), after nuclear staining with DAPI (**b**), and merged (**c**)

morphology and physiology [77, 79–81]. In the context of eye growth regulation and myopia, there seems little doubt that the RPE also serves as a relay or conduit for retina-derived growth modulatory signals directed at the choroid and sclera. It is thus also highly likely to play a critical role in the eye growth regulation and refractive error development, although the possibility has been the focus of attention only recently and thus this field of knowledge is still evolving [72].

Growth Factors and Eye Growth Regulation

The RPE is the source of a wide variety of growth factors essential to the maintenance of the structural integrity and functions of retina and choroid [81]. Pigment epithelium-derived factor (PEDF) and transforming growth factor-β (TGF-β) are among the growth and neurotrophic factors secreted predominantly from the apical (retinal) side, while vascular endothelial growth factor (VEGF) appears to be predominantly secreted from the basolateral (choroidal) side, with a presumed role in maintaining the survival and fenestrated morphology of the choriocapillaris [77, 79, 82–84]. The fact that the RPE also expresses receptors for a number of growth factors implies also direct autocrine regulation of some of its functions [85–87]. Some growth factors are also likely to contribute to pathological changes, as the RPE is known to respond to changes and injuries by differentially modulating the synthesis and secretion of certain ones [88, 89]. In the context of eye growth regulation and myopia, there has been only limited study of the roles of RPE-derived growth factors. Nonetheless, a number of multifunctional growth factors, including TGF-βs, bone morphogenetic proteins (BMPs), basic fibroblast growth factor (bFGF), and insulin-like growth factors (IGFs) have been investigated to this end.

TGF-βs

TGF-βs are probably the most studied growth factors in myopia research. They belong to a superfamily of structurally-related, multifunctional growth factors; apart from isoforms of TGF-β, it also includes BMPs, growth differentiation factors (GDFs), activins, inhibins, nodal and anti-Müllerian hormone (AMH) [90, 91]. Outside the eye, TGF-βs are known to be secreted by many cell types and involved in a wide variety of physiological and pathological processes, including embryonic development, organogenesis, immune modulation, cancer progression, wound healing and extracellular matrix remodeling [90, 91]. TGF-β has three isoforms, TGF-β1, TGF-β2, and TGF-β3; all are secreted as dimeric precursor proteins, from which active TGF-β is subsequently released in target tissues [92]. The known molecules involved in TGF-β activation include matrix metalloproteinase 2 (MMP2), MMP9, thrombospondin 1 (THBS1), and integrin [92–95]. Upon ligand binding to TGF-β receptors, intermediate steps involving the formation of heterotetrameric receptor complexes and then phosphorylation of receptors lead to activation of down-stream canonical or non-canonical signaling pathways to induce expression of target genes [90, 96].

Investigations into the roles of TGF-βs in eye growth regulation and myopia encompass both chick and mammalian models and different visual manipulations, with focuses on changes in either or both gene and protein expression of TGF-βs and/or their receptors in a variety of ocular tissues [97–109]. Scleral fibroblasts have been the focus of a number of such *in vivo* studies, as well as *in vitro* studies, which have linked TGF-β to cell proliferation, synthesis and secretion of proteoglycans, collagen production, cell contraction and cell phenotype alteration [100, 101, 110–112]. In terms of investigations into the ocular growth effects of exogenous TGF-β in animal models, one involving the chick form-deprivation model found subconjunctival injection of TGF-β1 to inhibit the myopia rescue effect of bFGF [113].

Chick, tree shrew and marmoset models have all been used in investigations into the role of RPE-derived TGF-β on eye growth regulation. Key results from relevant studies involving TGF-β isoforms and RPE are summarized in Table 7.1. In chicks, mRNA for all three TGF-β isoforms and all three TGF-β receptors (TGFBR1, TGFBR2, TGFBR3), was found to be expressed in the RPE, which also showed isoform-specific, defocus-sensitive changes in TGF-β gene expression [109]. Specifically, short-term exposure to myopic defo-

Table 7.1 Summary of key findings in animal models of eye growth regulation in relation to TGF-β expression changes in RPE alone, or combined with adjacent tissues

Animal	Visual treatments	Ocular tissues	TGF-β isoforms	Methods	Main results	References
Chick	FD for 12 days	Retina-RPE-choroid	TGF-β2	Protein (ELISA)	↑	Seko et al. [107]
Chick	FD for 10 days	Retina-RPE-choroid Retina-RPE-choroid	TGF-β1 TGF-β1, 2, 3, 5	tRNA (PCR) Protein, active form (WB)	↓ ↓	Honda et al. [100]
Chick	+7 or −7 D lens for 15, 30, 120 min	Retina-RPE	TGF-β2	mRNA (qPCR)	NS	Simon et al. [108]
Chick	−10 or +10 D for 2 or 48 h	RPE	TGF-β1, 2, 3	mRNA (qPCR)	NS with −10 D ↑TGF-β2 with +10 D	Zhang et al. [109]
Tree shrew	−5 D lens for 6 or 24 h	RPE Retina-RPE	TGF-β1, 2 TGF-β1, 2	mRNA (qPCR) mRNA (qPCR)	↓TGF-β2 24 h NS	He et al. [114]

FD Form-deprivation, *WB* Western blot, *NS* no significant change in treated versus control, ↑ increase in treated versus control, ↓ decreased treated versus control

cus (e.g., with +10 D lenses) resulted in selective up-regulation of TGF-β2, up to 3.5- and 7.5-fold after 2 and 48 hours exposures, respectively. The 2 hours short-term treatment was used to identify genes important for the initiation of altered eye growth, before detectable changes in eye growth can occur, while genes showing differential expression with the 48 hours treatment are likely involved in maintaining the altered growth pattern, as by this time altered growth is detectable with biometry. As imposed myopic defocus inhibits ocular growth, these results suggest that RPE-derived TGF-β2 serves as an inhibitor of ocular elongation. In the broader context of myopia control, they identify TGF-β2 as a gene of interest. In the tree shrew, 24 hours of −5 D lens resulted in a 1.4-fold down-regulation of TGF-β1 gene expression in RPE, but had no effect on TGF-β2 [114]. Interestingly, no differential gene expression of TGF-β1 was observed in combined retina-RPE tissues in the same study, perhaps reflecting opposing retinal expression changes. Nonetheless, differential gene expression of TGFβ-induced (TGFBI) in retina-RPE combined samples was detected in a microarray study involving marmosets [115].

Direct supporting evidence for ocular growth inhibition by RPE-derived TGF-β is still lacking, in part due to design features of relevant earlier studies that render their results inconclusive. Specifically, TGF-βs are secreted proteins that exist in both latent and active forms, yet these properties were not generally taken into account in previous studies in the choice of protein expression assays, which were also mostly undertaken on combinations of ocular tissues, as were gene expression measurements [100, 102, 107, 108].

BMPs

The roles of BMPs in eye growth regulation and myopia development have been the subject of a number of recent investigations. As already noted, BMPs belong to the TGF-β superfamily. As the largest subfamily of this superfamily, it contains more than 20 members, which are typically further grouped into four subfamilies, based on their sequence similarity and known functions [116, 117]. Their name—bone morphogenetic protein—reflects their initial recognition as proteins involved in ectopic bone formation, although BMPs are now known to play important roles in many biological events, including embryogenesis, postnatal homeostasis, stem cell regulation and regeneration, as well as in various pathological events, including neovascularization and some cancers [88, 118–123]. BMPs are synthesized as precursor proteins, which are then secreted in dimeric form with or without their prodomain, or packaged into vesicles for release from cells [124]. Secreted BMPs may interact directly with neighboring cells, be released into the bloodstream, or bind with extracellular antagonist proteins, such as noggin, which regulate their activity. BMPs signal through two different types of serine/threonine kinase receptors, type I and type II receptors, with Smad proteins playing an important intracellular role in the transduction to the nucleus of signals from activated receptor complexes [118, 124].

During embryonic development, BMPs and their receptors are widely expressed in ocular tissues, with diverse roles, including lens induction, ciliary body morphogenesis, RPE specification, retinal patterning, retinotectal projection and retinal stem cell differentiation [125–137]. In the postnatal eye, BMPs play important roles in maintaining physiology hemostasis and they have also been implicated in a number of ocular diseases [25, 88, 121, 122, 138–140]. To-date, expression patterns for BMPs and BMP receptors have been described for three commonly used myopia animal models—chicks, tree shrews and guinea pigs [99, 141–146]. Consistent with roles in ocular growth regulation, BMP gene and/or protein expression changes have been detected in one or more posterior ocular tissues in response to commonly used visual manipulations in all three models [99, 141, 142, 145–149]. Of potentially greater relevance to eye growth regulation, BMP2 has been found to induce the gene expression of four inhibitor of DNA binding proteins (IDs) in cultured chick scleral fibroblast; it also influences cell proliferation and extracellular matrix (ECM) synthesis and degradation, with reported effects in a cultured human scleral fibroblast model on collagen, glycosaminoglycan (GAG), and aggrecan

synthesis, as well as MMP2, TIMP2 and chondrogenesis-associated gene expression [142, 150–152]. Interestingly, increased BMP2 gene expression in human sclera fibroblasts was also found in response to mechanical loading, to which intraocular pressure contributes *in vivo* [153]. Collectively, these observations suggest a key role for BMP2 in scleral remodeling and thus in eye growth regulation.

The most direct evidence implicating BMPs in the eye growth modulating signal pathways linking the retina with the choroid and sclera comes from studies involving chick and tree shrew models [114, 143, 145, 146]. Key results for relevant BMP gene expression studies involving the RPE are summarized in Table 7.2. In relation to the RPE, defocus, sign-dependent differential regulation of BMP gene expression has been described in response to short-term negative or positive lens treatments in chicks [145, 146]. Changes in BMP2 gene expression are most robust although two other members of the BMP family, BMP4 and BMP7, also show similar bidirectional changes in gene expression, in accord with the direction of experimentally-induced eye growth changes. Specifically, expression is down-regulated in eyes showing accelerated growth, as in myopia progression, and up-regulated in eyes showing slowed (anti-myopia) growth. Other related gene expression studies did not find any defocus-induced changes in the retina and reported only small-scale changes in BMP gene expression in choroid relative to changes in RPE [144]. These observations together strongly suggest critical roles of RPE-derived BMPs in defocus-driven eye growth regulation, with BMPs serving as growth inhibitors. They also offer additional insight into the results of two other chick studies that examined gene expression in combinations of ocular tissues that included RPE. In one of these studies involving retina-RPE, BMP2 gene expression was reported to be down-regulated after 6 hours and 3 days of form-deprivation, in the same direction as that induced by negative lenses, with both treatments leading to accelerated eye growth [143]. The other study involving retina-RPE-choroid reported early, bidirectional BMP2 gene expression under conditions inducing myopia and hyperopia, and as described above for isolated RPE [154]. While similar roles for RPE-derived BMPs in eye growth regulation in mammals await confirmation, a recent study involving tree shrews reported down-regulation of BMP2 gene expression in retina-RPE after 24 hours of −5 D lens treatment [114]. Recent confirmation of BMP2 expression in normal guinea pig RPE (unpublished observation), also represents a promising first step in a line of research that has

Table 7.2 Summary of key findings in animal models of eye growth regulation in relation to BMP expression changes in RPE alone, or combined with adjacent tissues

Animal	Visual treatments	Ocular tissues	BMPs	Methods	Main results	References
Chick	FD for 6 h or 3 days	Retina-RPE	BMP2	mRNA (microarray, qPCR)	↓	McGlinn et al. [143]
Chick	−15 or +15 D for 6 h or 3 days	Retina-RPE	BMP2	mRNA (microarray, qPCR)	↓ with −15 D	Stone et al. [155]
Chick	−10 or +10 D for 2 or 48 h	RPE	BMP2	mRNA (qPCR)	↑ with +10 D ↓ with −10 D	Zhang et al. [146]
Chick	−10 or +10 D for 2 or 48 h	RPE	BMP4, 7	mRNA (qPCR)	↑ with +10 D ↓ with −10 D	Zhang et al. [145]
Chick	−10 or +10 D for 1, 2, or 3 days	Retina-RPE-choroid	BMP2	mRNA (RNA sequencing)	↑ with +10 D ↓ with −10 D	Riddell et al. [154]
Tree shrew	−5 D lens for 6 or 24 h	RPE Retina-RPE	BMP2, 4 BMP2, 4	mRNA (qPCR) mRNA (qPCR)	NS ↓	He et al. [114]

FD Form-deprivation, *WB* Western blot, *NS* no significant change in treated versus control, ↑ increase in treated versus control, ↓ decreased treated versus control

potential to open up new therapeutic avenues for myopia control.

Other Growth Factors

In addition to TGF-βs and BMPs, two other growth factors that have also attracted the interest of myopia researchers are IGFs and bFGF. Currently, relatively little is known about their signaling pathways, including whether or not the RPE is an important player, either as a site of synthesis or as a target. Nonetheless, studies that offer some evidence implicating the RPE are summarized below.

Insulin-like growth factors (IGFs) are polypeptide growth factors of the insulin family that consists of two members, IGF1 and IGF2, which play important roles in development and diseases [156–158]. Both IGFs and IGF receptors (IGF1R and IGF2R) are also widely expressed in ocular tissues [159–162]. For example, in chicks, IGF receptors have been detected in all posterior ocular tissues, i.e., retina, RPE, choroid, and sclera [161, 162]. IGFs and their receptors have also been implicated in altered eye growth in animal models. For example, in chicks, IGF1R is reported to be differentially regulated in RPE after 4 hours of positive lens wear and intravitreal injection of IGF1, to induce myopic eye growth [161–163]. Differential regulation of IGF2 was also observed in retina-RPE in tree shrews, after 24 hours of negative lens wear, although no change in IGF1 gene expression was detected [114]. Results from *in vitro* studies involving cultured human RPE cells also support a role for the RPE in IGF-mediated eye growth regulation; they were found to express both IGFs and their receptors and also secrete IGFs into the culture medium [164–166].

RPE is one of a number of ocular tissues that synthesize bFGF and its receptors and also secrete bFGF [167]. The retina and choroid were among other ocular tissues found to express bFGF and its receptors in one study in chicks, although due to the technique used and the dense pigmentation of the RPE, it was not possible to characterize bFGF receptor expression profile in this study [168]. Since both intravitreal and subconjunctival injections of bFGF are effective in inhibiting the excessive eye elongation induced by form-deprivation, the retina, RPE, choroid and sclera all represent potential sites of action [113, 169]. Other indirect evidence supporting a role for bFGF as an important regulator of eye growth includes an observed decrease in bFGF in the chick sclera after 2 weeks of form-deprivation. However, that no change in bFGF was found in combined retina-RPE-choroid in the same study tends to argue against the RPE as the source of bFGF [107]. Results of other studies involving guinea pigs also tend to point away from the RPE. For example, inhibition of lens-induced myopia with a peribulbar injection of bFGF has been linked to altered scleral expression of both collagen and integrin and expression of bFGF was reported to be reduced in scleral desmocytes isolated and cultured from experimentally (lens)-induced myopic eyes [110, 170]. Thus, while these results in chicks and guinea pigs are consistent with a role for bFGF as a "stop" signal in growing eyes, the exact details of the growth modulating signal pathway, including the identity of the cells mediating the action of bFGF, await further study.

Neurotransmitters and Eye Growth Regulation

A number of neurotransmitters have been implicated in eye growth regulation and the RPE is known to both express receptors for some of these neurotransmitters and also synthesize and secrete some of them [72, 171]. Therefore it is possible that the RPE is involved in neurotransmitter-related eye growth regulation and myopia development. Retinal neurotransmitters, including dopamine (DA), acetylcholine (Ach), glucagon and vasoactive intestinal peptide (VIP) are the most studied of these molecules in myopia research. Related studies targeting DA and Ach are reviewed below.

Dopamine

Dopamine is one of the most studied neurotransmitters in the context of eye growth regulation and myopia development [172–174]. Dopamine

is widely expressed in both the central nervous system and retina where it plays important roles in development. In the postnatal retina, dopamine has been linked to retinomotor movements and the uncoupling of gap junctions on horizontal cells, both of which affect visual sensitivity [175–177]. Dopaminergic receptors represent a large family of G protein-coupled receptors (D1–D5), with members grouped into two subfamilies, D1-like (D1, D5) and D2-like (D2–D4) receptors, based on their biochemical and pharmacological properties [178]. Evidence supporting the role of dopamine in eye growth regulation comes mainly from two lines of research showing that: (1) retinal levels of dopamine and its metabolites are decreased in animals with visual manipulations that accelerate eye growth, and (2) locally administered exogenous dopamine receptor agonists inhibit the excessive eye elongation that underlies myopia, with more recent studies involving wild type, and genetically modified mice models offering further confirmatory evidence [52, 173, 179–197]. The evidence linking reduced dopamine levels and turnover with myopia comes from studies involving young chicks, guinea pigs, tree shrews and monkeys, which report consistent reductions with form-deprivation or lens induced models [181, 183, 187, 188, 196]. Further indirect evidence tying these changes to eye growth comes from studies of eyes allowed to recover from form-deprivation myopia, when both retinal dopamine and DOPAC levels return to levels comparable to those of contralateral control eyes [198]. Interestingly, low vitreal concentrations of dihydroxyphenylacetic acid (DOPAC), a dopamine metabolite, have also been linked to the use of low light intensity in rearing and associated myopia development in chicks [199]. Studies involving chicks, guinea pigs, tree shrews and monkeys are also the source of supporting evidence that the development of myopia can be inhibited by dopamine agonists, delivered by either intravitreal or subconjunctival injection, or by topical application [180, 184–186, 189–192, 194, 195, 197]. While retinal dopamine receptors are potential sites of action for the latter effects, the RPE is also known to express both D1- and D2-like receptors [176, 200, 201]. Furthermore, the RPE appears to be a readily accessible target for applied drugs. For example, both intravitreal and subconjunctival injections of radioactively-labeled spiperone, a D2 receptor antagonist, were found to reach the RPE [192]. That the RPE is a plausible site of action for dopaminergic effects on eye growth is further supported by other *in vitro* studies. One such study reported physiological responses, including hyperpolarization, in response to dopamine applied to either apical or basolateral membranes of cultured RPE [202]. In another *in vitro* co-culture study, apomorphine, a dopamine receptor agonist, dramatically inhibited the growth-stimulatory effect of RPE cells on scleral chondrocytes [203]. Nonetheless, there remain many unanswered questions in relation to the signal pathway mediating the anti-myopia action of dopamine, given that in yet two other studies, RPE was found to both synthesize and secrete dopamine [204, 205].

Acetylcholine (Ach)

Acetylcholine is a ubiquitous yet important neurotransmitter with critical roles in retina development and functions [206, 207]. Retinal cholinergic cells comprise several subsets of amacrine cells, which are known to synapse with other neurotransmitter networks in the retina, including dopaminergic cells [208–210]. Acetylcholine (Ach) receptors fall into two broad categories, muscarinic acetylcholine receptors (mAChRs) and nicotinic acetylcholine receptors (nAchRs) [211, 212]. Muscarinic receptors (mAChRs), which are widely distributed throughout the eye, represent a family of G protein-coupled receptors, with five receptor subtypes (M1–M5) described in mammals [211, 212]. Consistent with the reports of mAchR on RPE as well as in retina, *in vitro* studies have shown that intracellular signal pathways in RPE can be activated by mAchR agonists [206, 209, 213–216]. Different from the finding with dopamine of reduced retinal turnover in form-deprived myopic eyes, levels of retinal Ach and its metabolite, choline, appear unaffected by the development of myopia in both chicks and tree shrews [188]. On the other hand, intervention studies have shown antimuscarinic drugs to be effective inhibitors of myopia development in both humans and animal models. In animal stud-

ies, drugs were administrated via either intravitreal or subconjunctival injection, while topical drops have been the norm for human studies, which have also been limited to atropine, a non-selective antagonist, and pirenzepine, a M1-receptor antagonist [195, 217–225]. Reports in more recent human studies of inhibitory effects with much lower concentrations of topical atropine than used in earlier studies (e.g., 0.01 and 0.1% compared to 1%) [42], argue against inner ocular tissues, including the retina and RPE, as sites of action, based on pharmacokinetic principles. The relatively high intravitreal doses needed to achieve treatment efficacy in another comparative study of eighteen antimuscarinic drugs tested in form-deprived chicks, also challenges the notion of a muscarinic receptor-mediated mechanism involving retina or RPE [226], leading to more recent speculation on possible nonmuscarinic mechanisms being involved [227–229]. These conclusions are also in line with findings from other studies, including *in vitro* ones, pointing to the choroid and/or sclera as likely site(s) of action for the anti-myopic effects of antimuscarinic drugs, although underlying mechanisms remain poorly understood [217, 230, 231].

As a final aside, it should be noted that intravitreally-injected nAchR antagonists have been shown to influence eye growth in chicks [232]. As nAchRs have also been found in retina and RPE, they are plausible sites mediating the complex response patterns observed.

Glucagon and Vasoactive Intestinal Peptide (VIP)

Glucagon and vasoactive intestinal peptide (VIP) are part of a superfamily of secretin-glucagon peptides that function as neurotransmitters or neuromodulators in both central and peripheral nervous systems, acting through G-protein coupled receptors [233]. Both glucagon- and VIP-immunoreactive neurons have been described in chick retina [234–236]. Furthermore, the RPE also expresses glucagon receptor mRNA, and both glucagon and VIP have been shown to stimulate intracellular activities in RPE [237–240].

Evidence for roles of glucagon in eye growth regulation comes largely from studies in chicks. In the earliest of such studies, the expression of the immediate-early gene, ZENK, was reported to be decreased in glucagonergic amacrine cells in response to both form-deprivation and negative lens treatments and increased with positive lens treatments in chicks [241]. Later studies reported retinal glucagon mRNA to be also down-regulated with negative lens treatments and up-regulated with positive lens treatments [237, 242]. In addition, differential regulation of retinal mRNA levels of preproglucagon was found with form-deprivation and negative lens treatments [237, 243]. Finally, retinal glucagon peptide levels were found to be decreased after exposure to negative lenses [244]. That glucagon may act as a stop signal for eye growth, as suggested by these studies, is also consistent with results of pharmacological studies in which glucagon agonists, injected intravitreally, were found to inhibit experimentally induced myopia [244–246]. It is also plausible, but not conclusively established, that the RPE is the target for retinal and exogenous glucagon, serving as a signal relay for these ocular growth effects.

VIP has been the subject of far fewer studies, with one reporting levels of VIP to be significantly increased in the retinas of form-deprived monkeys and another reporting VIP gene expression to be up-regulated in tree shrew retina and retina-RPE, while down-regulated in RPE with negative lens wear [114, 247, 248]. VIP receptor antagonists, delivered by intravitreal injection, have also been reported to inhibit form-deprivation myopia in chicks [249]. However, neither the role of VIP nor the role of glucagon in form-deprivation myoia has been confirmed in the mouse model [250].

Ions, Ion Channels and Eye Growth Regulation

The RPE plays important roles in regulating the ion composition and volume of the subretinal space and choroid and thus the maintenance of tissue homeostasis. As already noted, the RPE comprises a monolayer of highly polarized cells interconnected with tight junctions, which restrict the paracellular movement of ions and fluid. That the paracellular resistance between RPE cells is ten

times higher than the transcellular resistance is one measure of the effectiveness of these junctions. The polarized expression of ion channels and other functionally related molecules on RPE has been well documented and is consistent with the unidirectional fluid transport across the RPE, in an apical-to-basal direction, facilitated by the transport of ions [75, 80, 81, 251]. Thus Na^+-HCO_3^- and Na^+-K^+-$2Cl^-$ cotransporters, along with Na^+-K^+-ATPase, which are found on the apical membranes of the RPE, allow the intracellular uptake of Cl^- and regulation of intracellular pH. Cl^- ions along with K^+ ions are extruded into the choroid from ion channels on the basolateral membranes of the RPE, which also express the cystic fibrosis transmembrane conductance regulator (CFTR) [81, 252]. This movement of ions serves to drive the movement of water from the subretinal space into the choroid.

Investigations into the roles of ion channels in eye growth regulation and the possible involvement of RPE have been limited to the chick model [69, 72]. In eyes made myopic by form-deprivation, both retinal and choroidal tissues were found to have markedly raised Na^+ and Cl^- levels, with K^+ levels also elevated, albeit localized to the outer retina-RPE region [253]. In eyes allowed to recover from induced myopia, the levels of K^+, Na^+, and Cl^- normalized around the time of refractive error recovery, while during the recovery phase, the retina, RPE, and choroid all showed thickening and edema [253, 254]. These findings point to potential roles of ion and fluid movement across RPE in refractive error regulation, with more direct evidence contained in related pharmacological studies. Specifically, intravitreal injection of barium chloride, a non-specific potassium channel inhibitor, was found to inhibit the compensatory ocular growth responses to imposed optical defocus, irrespective of its sign, while bumetanide, a selective Na^+-K^+-Cl^- cotransporter inhibitor, selectively inhibited the response to negative lenses [255]. In addition, an unrelated study reported differential regulation of several Cl^- transporters and channels in the RPE with negative lenses, with gene and protein expression being down-regulated after just one day of lens wear [256].

The identity of the signal molecules mediating the above changes in ion transport and fluid movement across the RPE during the development and recovery from myopia remains unresolved, although two *in vitro* studies point to dopamine and Ach as plausible candidates. In one of these studies, using retina-RPE-choroid preparations, dopamine was found to modulate basal membrane Cl^- conductance in chick RPE [202], and in the other study using human RPE cultures, mAchR agonists were found to induce rapid increases in intracellular calcium [213].

Morphological Changes in RPE in Experimental Myopia

In relation to myopic growth-related morphological changes in RPE, two studies of relevance include one involving chicks and another, quokka wallabies, which are a marsupial [257, 258]. As one of three layers lining the scleral cup, which undergoes substantial enlargement in myopia, the RPE must necessarily undergo substantial expansion of its surface area in parallel. In chicks made myopic by form-deprivation for 1–2 weeks, individual RPE cells were found to thin and stretch to maintain coverage of the expanding vitreous chamber, rather than dividing to add cell numbers; nonetheless, their hexagonal shapes were preserved [258]. Similarly in form-deprived quokkas, RPE cells were found to be enlarged in treated eyes, with their appearance being otherwise relatively unaffected, when compared against those of fellow, control eyes [257]. A unique finding of the latter study was the redistribution of multinucleated RPE cells in enlarged form-derived eyes, from the usual, mostly ventral location as seen in untreated (control) eyes, to more peripheral dorsal and nasal locations, around the retinal rim. However, it should also be noted that unlike most mammals and primates, the eyes of quokkas also grow throughout life, Nonetheless, these limited observations suggest that the RPE adapts to the expanding vitreous chamber in myopia, mostly through passive stretch, with possible implications for the long-term health of RPE cells and risk of pathology,

especially in highly myopic eyes [45]. Finally, it is noteworthy that application of mechanical stress to cultured RPE cells has been reported to induce VEGF in rat RPE and MMP-2 activation in human RPE [259, 260]. These observations raise the possibility that the mechanical forces experienced by the RPE of growing eyes may themselves indirectly influence eye growth through effects on RPE activity.

Summary

In summary, the RPE likely plays an important role in local eye growth regulation and thus the development of myopia, reflecting in part its critical location, interposed between the retina and choroid. Observations of growth factor synthesis and secretion, neurotransmitter receptor expression and activation, ion exchanges and fluid movement across RPE, are also compatible with a role for the RPE in eye growth regulation. Further elucidation of the presumed eye growth modulatory signal pathways and the role of the RPE as a signal relay may lead to novel therapeutic interventions for myopia control. An improved understanding of the morphological and functional changes in RPE at various stages of disease development and the key mediating factors may also lead to improved management of the pathological complications of myopia, including myopic maculopathy.

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