Published in final edited form as:
Prog Retin Eye Res. 2008 November ; 27(6): 579–595. doi:10.1016/j.preteyeres.2008.08.002.

Epithelial phenotype and the RPE: Is the answer blowing in the Wnt?

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Abstract

Cells of the human retinal pigment epithelium (RPE) have a regular epithelial cell shape within the tissue *in situ*, but for reasons that remain elusive the RPE shows an incomplete and variable ability to re-develop an epithelial phenotype after propagation *in vitro*. In other epithelial cell cultures, formation of an adherens junction (AJ) composed of E-cadherin plays an important early inductive role in epithelial morphogenesis, but E-cadherin is largely absent from the RPE. In this review, the contribution of cadherins, both minor (E-cadherin) and major (N-cadherin), to RPE phenotype development is discussed. Emphasis is placed on the importance for future studies of actin cytoskeletal remodeling during assembly of the AJ, which in epithelial cells results in an actin organization that is characteristically zonular. Other markers of RPE phenotype that are used to gauge the maturation state of RPE cultures including tissue-specific protein expression, protein polarity, and pigmentation are described. An argument is made that RPE epithelial phenotype, cadherin-based cell–cell adhesion and melanization are linked by a common signaling pathway: the Wnt/β-catenin pathway. Analyzing this pathway and its intersecting signaling networks is suggested as a useful framework for dissecting the steps in RPE morphogenesis.

Also discussed is the effect of aging on RPE phenotype. Preliminary evidence is provided to suggest that light-induced sub-lethal oxidative stress to cultured ARPE-19 cells impairs organelle motility. Organelle translocation, which is mediated by stress-susceptible cytoskeletal scaffolds, is an essential process in cell phenotype development and retention. The observation of impaired organelle motility therefore raises the possibility that low levels of stress, which are believed to accompany RPE aging, may produce subtle disruptions of cell phenotype. Over time these would be expected to diminish the support functions performed by the RPE on behalf of photoreceptors, theoretically contributing to aging retinal disease such as age-related macular degeneration (AMD). Analyzing sub-lethal stress that produces declines in RPE functional efficiency rather than overt cell death is suggested as a useful future direction for understanding the effects of age on RPE organization and physiology. As for phenotype and pigmentation, a role for the Wnt/β-catenin pathway is also suggested in regulating the RPE response to oxidative stress. Exploration of this pathway in the RPE therefore may provide a unifying strategy for advancing our understanding of both RPE phenotype and the consequences of mild oxidative stress on RPE structure and function.

1. Introduction

The retinal pigment epithelium (RPE) displays the anatomic features of a typical monolayer epithelium with tightly packed cuboidal to columnar cells and apical microvilli. RPE cells also have a notably regular hexagonal organization in surface view and numerous cytoplasmic granules, including the pigment granules that help give the tissue its name. Many details have

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been added to the morphologic description of the RPE since the tissue was discovered more than a century ago (Wolfensberger, 1998), but a challenge that still remains is to determine how this characteristic RPE phenotype comes about.

The focus of this review is on whether and how the RPE cell reacquires a mature epithelial architecture following disruption of a previously established monolayer. (For issues relating to RPE specification during embryogenesis see reviews by Bharti et al. (2006) and Martinez-Morales et al. (2004)). To keep the emphasis on what might be called *re*-morphogenesis, the term ‘maturation,’ ‘epithelialization’ or ‘epithelial morphogenesis’ is used in preference to the term ‘differentiation’, which is commonly used when discussing embryonic development. The ability of the RPE to undergo re-morphogenesis is of considerable interest to both cell biologists and ophthalmologists. The former want to understand the molecular pathways that regulate cell-type-specific phenotypes, and the latter want to find ways to restore the structure of an RPE layer that is disrupted by injury or disease so that the tissue remains competent to fully support the retina.

A related topic that will also be discussed is whether normal aging reduces the organizational state of RPE cells. The RPE is believed to play a central role in retinal diseases of aging and many investigations have been conducted to examine RPE cell loss with age. Yet the hallmark of aging is gradual functional declines that take place over years rather than widespread cataclysmic cell death. Little is known, however, about the structure of the aging RPE or the mechanisms whereby age could cause phenotypic changes in the tissue. Even subtle modifications of cell shape or sub-cellular organization could have profound consequences for the efficient function of the tissue over time, and ultimately for the ability of the RPE to support the survival of adjacent photoreceptors.

In addressing the topic of phenotype development as it relates to the RPE, the role of cadherin adhesions is discussed in some detail, but the main goal here is to focus on the big picture of epithelial morphogenesis. Many rapidly advancing research fields, such as those relating to cell adhesion, sub-cellular molecular trafficking, tumor progression, oxidative stress and aging, are relevant for understanding epithelial cell phenotype development and retention. The intersection of these fields is providing an increasingly complicated and integrated view of epithelial cell shape determination. The intent here is to discuss how the RPE fits into this picture in an attempt to identify gaps in our understanding of the morphogenesis of this epithelial cell type, and perhaps to provide a framework for filling in those gaps. Selected concepts are invoked from several broad research areas inside and outside of ocular cell biology. Whenever possible, comprehensive recent reviews on those topics are cited rather than primary references to help direct the reader to the relevant literature.

2. Monolayer epithelial cell phenotype and the RPE

The defining morphologic feature of monolayer epithelial cells such as the RPE is compartmentalization of the cytoplasm along an apico-basal axis. A well-developed monolayer epithelium has organelles and cytoskeletal elements localized to specific sub-cellular positions. It also has a characteristic apical zonular band of cell–cell adhesion proteins associated with a prominent circumferential actin microfilament bundle, and plasma membrane molecules polarized to cell-type-specific apical, lateral or basal domains.

How epithelial cell topography is established and maintained has been the topic of many investigations, with the field skyrocketing in the latter part of the 1980's and early 1990's. At that time the major models for dissecting the events of epithelial morphogenesis were epithelial cell lines primarily from kidney and gut. Using these lines, development of an epithelial phenotype was shown to be critically dependent upon interactions between the actin microfilament and intermediate filament systems and the cell surface at sites of cell–substrate

and cell–cell attachment (Hitt and Luna, 1994; Luna and Hitt, 1992; Rodriguez-Boulan and Nelson, 1989). Fully dissociated epithelial cells lack the epithelial trait of apico-basal polarity. Attachment to surfaces and to one another provides cues that discriminate apical membrane from basolateral, and that trigger remodeling of sub-cellular architecture (Nelson, 1991; Wang et al., 1990; Wollner et al., 1992; Wollner and Nelson, 1992).

One of the most extensively studied proteins used in earlier investigations of epithelial polarity was Na/K ATPase, the sodium pump. This ion transporter plays a central role in maintaining the sodium gradient responsible for the transport of virtually all nutrients and metabolites (Jaitovich and Bertorello, 2006). In epithelial monolayers Na/K ATPase is enriched to a cell-type-specific domain as required to mediate vectorial transport across the tissue. The mechanisms governing pump polarization were studied most intensively in the canine kidney-derived cell line MDCK where the pump is basolateral. Pump polarity is achieved by a complex sequence of events involving both targeting to and selective retention at the baso-lateral cell surface (Gottardi and Caplan, 1993; Hammerton et al., 1991; Mays et al., 1995; McNeill et al., 1990; Nelson, 1991; Nelson and Veshnock, 1987; Wollner and Nelson, 1992; Zurzolo and Rodriguez-Boulan, 1993).

In contrast to the monolayer epithelial cell models used to investigate Na/K ATPase polarity, the sodium pump is enriched in the opposite or apical membrane domain of RPE cells (Bok, 1982; Gundersen et al., 1991; Okami et al., 1990; Rizzolo, 1990; Steinberg and Miller, 1979). It was this ‘reverse polarity’ of the RPE that brought this cell type to the attention of the broader cell biology community and ushered in an era of high interest in epithelial morphogenesis of the RPE.

2.1. Human RPE cell cultures as a model for epithelial morphogenesis

Cells in culture are indispensable for investigating the mechanisms governing epithelial cell maturation, including protein polarization, but they have inherent weaknesses for modeling cellular behavior *in situ*. Cultures originating from the RPE have both benefits and disadvantages. The tissue is largely non-mitotic *in situ*, but the cells can be readily propagated *in vitro* even from aged human donors. However, RPE cultures appear to have a limited capacity to recapitulate epithelialization to achieve a high state of phenotypic maturation.

Despite their limited epithelial maturation (or perhaps because of it), RPE cultures may nonetheless be well suited for investigating whether and how RPE cells undergo re-morphogenesis. Growing cells in culture from explanted tissues inevitably involves disruption of pre-formed cell layers. Even RPE cultures of fetal origin are propagated from tissues that had previously differentiated into pigment epithelium prior to tissue isolation. Therefore studying morphogenesis taking place in RPE cell cultures may be particularly useful for probing the competence of the RPE to re-establish tissue-level architecture after the normally quiescent epithelium is disturbed. The relevance of this issue is illustrated by considering what might happen to the RPE damaged by retinal detachment. The desired outcome of detachment repair is a restored monolayer of contiguous cells with their phenotype re-established. Unfortunately, the outcome can be proliferative vitreoretinopathy (Agrawal et al., 2007; Asaria and Charteris, 2006), a disorder in which the RPE is hyperplastic and migratory with a phenotype that is far from epithelioid.

2.2. Mechanisms of morphogenesis in other monolayer epithelial cells: central role of cell–cell adhesions

Epithelial phenotype development *in vitro* is initiated on cell–cell contact and proceeds as cultures become more tightly packed at confluency and post-confluency. The triggering events in morphogenesis were ascribed in the 1990's to the formation of the calcium-dependent cell–

cell adherens junction (AJ) (Nathke et al., 1993; Takeichi, 1991, 1995). At this time, the major binding elements of the AJ were identified as members of the cadherin family of adhesion molecules (Geiger and Ayalon, 1992; Takeichi, 1995), especially E-cadherin in most epithelial cells (Rodriguez-Boulan and Nelson, 1989; Takeichi, 1991). Cell contact initiates the time-dependent formation of cadherin-mediated adhesions during which the cytoplasmic domain of the cadherin molecule forms a complex with proteins that mediate association with the actin cytoskeleton (Gumbiner and McCrea, 1993; Hinck et al., 1994; Nathke et al., 1993), with the major linkage function attributed to members of the catenin family (α-catenin, β-catenin, plakoglobin). When cell contacts first form, E-cadherin is readily solubilized in buffers containing Triton-X-100, but it later becomes resistant to detergent extraction and localized to junctional sites (Hinck et al., 1994; McNeill et al., 1993). As the formation of cadherin adhesions progresses, actin filaments organize into circumferential bundles to produce an adherens junction that is zonular in epithelial cells (Yonemura et al., 1995). E-cadherin junctions can induce remodeling of the cell surface membrane cytoskeleton and associated plasma membrane molecules (such as Na/K ATPase (McNeill et al., 1990) leading to the development of distinct apical, lateral and basal membrane domains (Nathke et al., 1993; Rodriguez-Boulan and Nelson, 1989).

This basic model of E-cadherin induction of an epithelial phenotype developed nearly two decades ago still stands, although it has been considerably updated recently. Two useful reviews summarize current evidence regarding how cadherins, catenins and their binding partners or regulatory molecules participate in multiple biological functions, including tissue morphogenesis (Halbleib and Nelson, 2006; Perez-Moreno and Fuchs, 2006). In forming complexes with catenins, the cytoplasmic domain of E-cadherin directly binds β-catenin and p120-catenin. β-Catenin is a key regulator of cadherin adhesive function (Daugherty and Gottardi, 2007) together with p120-catenin, which modifies the F-actin cytoskeleton and functions to stabilize cadherins in the membrane (Davis et al., 2003). In addition to binding cadherins, β-catenin also has a binding domain for α-catenin, which in turn binds cytoskeletal actin. Because of its binary binding function, α-catenin was believed for many years to directly interlink cadherins to the actin cytoskeleton. However, recently a more complicated picture has emerged in which α-catenin does not simultaneously bind both β-catenin and F-actin to provide a direct cadherin-to-actin linkage (Drees et al., 2005; Yamada et al., 2005). Rather α-catenin in the form of cytosolic homodimers regulates the local actin cytoskeleton at the forming AJ to promote reduced actin polymerization and the formation of unbranched actin cables oriented parallel to membranes. The combined actions of p120-catenin and α-catenin modulate multiple actin-binding partners or effectors including Arp2/3, Rho GTPases, afadin and formin-1 to produce cadherin clustering and actin rearrangements at nascent AJs (Perez-Moreno and Fuchs, 2006). Recruitment of additional actin-binding proteins including α-actinin, vinculin and myosin II to the maturing junction of monolayer epithelial cells culminates in the formation of the tension-generating circumferential actin microfilament bundle that characterizes an epithelial phenotype. Collectively these recent investigations on the cadherin–catenin system point to a central role of cytoskeletal rearrangements in epithelial junction formation, maturation and maintenance (Miyoshi and Takai, 2008).

The formation of cadherin-mediated adhesions, and thus phenotype development and retention, is also regulated by other aspects of cadherin accumulation, notably transport to membranes and protein turnover. In epithelial cells, E-cadherin transport to the cell surface requires binding to β-catenin (Chen et al., 1999) and retention at the cell surface is regulated by phosphorylation, notably of β-catenin and p120-catenin (Fukumoto et al., 2008; Maeda et al., 2006; Perez-Moreno and Fuchs, 2006; Wheelock and Johnson, 2003a, 2003b). Downregulation of cadherins from formed epithelial adhesions is accompanied by internalization then recycling or degradation of the cadherin, and by a disruption of cell phenotype.

Cadherins are not the only integral membrane proteins of AJs, nor is the AJ the only adhesion implicated in generating an epithelial phenotype. Upstream of the cadherin-based adhesion and responsible for the recruitment of cadherins to the AJ is the more recently identified nectin-based adhesion (Miyoshi and Takai, 2007; Ogita and Takai, 2006; Sakisaka et al., 2007). Downstream of the AJ and dependent upon the AJ for its establishment and maintenance is the occluding or tight junction (TJ) (Miyoshi and Takai, 2005).

Nectins are calcium independent adhesion proteins that in epithelial cells cooperate with components of both AJs and TJs to generate junctions that determine epithelial phenotype and polarity (Miyoshi and Takai, 2007; Ogita and Takai, 2006; Sakisaka et al., 2007). Nectins recruit E-cadherin to the forming AJ and interact directly or indirectly with many proteins enriched at junctional sites. An important nectin binding partner is the F-actin-binding protein afadin which mediates nectin linkage with the AJ protein α-catenin, and via α-catenin with additional components of the mature AJ (e.g., α-actinin and vinculin). Additionally, nectins regulate junction assembly by interacting with integrins, thereby mediating cross-talk between integrins and cadherins at adhesive sites (Ogita and Takai, 2008). Nectins also induce local actin rearrangements by activation of several actin effectors including cdc42, Rac, and afadin, the latter via activation of a key regulator of intercellular junctions, the small GTPase Rap1 (Kooistra et al., 2007; Sakamoto et al., 2006).

Nectins in epithelial cells also participate in the formation of tight junctions by a similar combination of mechanisms involving recruitment of TJ components through afadin, which binds the TJ adaptor protein ZO-1, and through regulation of actin dynamics by activation of cdc42. The TJ has three integral membrane proteins: claudins, occludins and junctional adhesion molecules (JAMs) (Miyoshi and Takai, 2005; Oliveira and Morgado-Diaz, 2007; Shin et al., 2006). The AJ and TJ form cooperatively but segregate into separate junctions in the mature apico-lateral adhesion complex of epithelial cells. TJs are particularly important for establishing epithelial cell polarity. Together with the AJ, the TJ comprises the structural barrier or fence that demarcates apical from baso-lateral membrane domains, an essential prerequisite for epithelial polarization. The TJ also acts as the regulated functional barrier that determines the paracellular permeability of an intact epithelial monolayer. Several other TJ-associated proteins that are involved in multiprotein complexes have been implicated in polarization of epithelial and other cell types including Crumbs, Pals1, PATJ, PAR-3, PAR-6 and aPKC.

Once established, the adhesions involved in epithelial morphogenesis are dynamic structures. It is their disassembly and re-assembly during tissue remodeling, repair and cancer that clarified the importance of adhesions for inducing and maintaining a mature epithelial phenotype. The loss of a once-formed epithelial phenotype, called an epithelium-to-mesenchyme transition (EMT), has been studied extensively especially in the contexts of normal development and tumor progression (Huber et al., 2005), and also in proliferative diseases of the eye involving the RPE (Saika et al., 2008). The hallmark of EMT is junction dysregulation, which leads to a loss of epithelial organization and the acquisition of a migratory state.

Literally thousands of studies have been performed to dissect the mechanisms of EMT, a complex process that has particular relevance for the topic of epithelial morphogenesis of the RPE. As indicated above, in culture models of other monolayer epithelial cell types E-cadherin plays a central role in inducing an epithelial phenotype. However, in RPE cultures, including from human donor tissue, the dominant cadherin is not E-cadherin. It is N-cadherin (Burke et al., 1999; Davis et al., 1995; McKay et al., 1997; Rak et al., 2006; Van Aken et al., 2003), a related type I classical cadherin that is not typically expressed by monolayer epithelial cells. Indeed, when expressed in epithelia N-cadherin is associated with EMT and loss of epithelial organization (Hazan et al., 2004; Islam et al., 1996; Maeda et al., 2005); the N-cadherin gene is included among the EMT signature group of genes which when expressed are implicated in

promoting a mesenchymal phenotype in carcinoma cells (Alonso et al., 2007; Andarawewa et al., 2007). Given that N-cadherin is abundantly expressed by the RPE, a legitimate question to ask is: how much of what is known about E-cadherin-induced epithelial morphogenesis applies to the RPE?

Discussing this question, and the broader issue of how the RPE phenotype comes about, requires a brief summary of the markers that are used to gauge the maturation state of an RPE monolayer *in vitro*.

3. Epithelial morphogenesis in human RPE cultures

3.1. Assessing morphogenetic state and markers of maturation

The developmental state of RPE cells *in vitro* is evaluated by gross morphology, by the expression of tissue-specific proteins, by the appropriate polarization of proteins known to be asymmetrically enriched in apical or baso-lateral domains, and by the ability of the cultures to perform tissue-level polarized functions.

Gross RPE cell phenotype is typically assessed by phase contrast microscopy, which shows a morphology at confluency that is often described as “epithelioid” or “cobblestone”. Other microscopic methods such as scanning or transmission electron microscopy show morphologic markers of epithelial organization in cultured RPE monolayers including apical microvilli (Anderson et al., 1990; Heth et al., 1987) or apico-lateral cell–cell junctions with their actin microfilament undercoat (Davis et al., 1995; Heth et al., 1987). While these features distinguish RPE cultures from fibroblastic cell types, they provide little concrete information about the extent of epithelialization.

With regards to protein expression markers for RPE cultures, the most commonly used are cytokeratin intermediate filament proteins, especially CK 8 and 18. Cytokeratins are readily detected in virtually all RPE cultures, but their presence indicates only that the cells are derived from an epithelium. More useful for evaluating RPE developmental state is determining whether the cells express genes or proteins that are specific for or highly expressed by the RPE. Commonly used markers in this category are cellular retinaldehyde binding protein (CRALBP) and RPE65, proteins that are involved in retinoid processing required for regeneration of visual pigments by photoreceptors. A more recently added identifier of RPE maturation is expression of bestrophin-1, a protein that plays incompletely understood roles in chloride conductance and calcium signaling (Marmorstein and Kinnick, 2007). Also occasionally used as RPE maturation markers are enzymes involved in melanogenesis, e.g., tyrosinase or tyrosinase-related proteins (Abul-Hassan et al., 2000; Ohno-Matsui et al., 2005; Rak et al., 2006), or the accumulation of pigment itself (Campochiaro and Hackett, 1993; Dorey et al., 1990; Gamm et al., 2008; Maminishkis et al., 2006; Ohno-Matsui et al., 2005; Pfeffer et al., 1986; Rak et al., 2006; Song and Lui, 1990). RPE65, CRALBP and/or bestrophin gene expression has been shown in RPE cultures derived from mammalian RPE, including both fetal and adult human RPE cells (Gamm et al., 2008; Kanuga et al., 2002; Maminishkis et al., 2006; Ohno-Matsui et al., 2005; Rak et al., 2006). Detection of the protein, however, is more limited. CRALBP protein has been found in cultures of adult human RPE under limited culture conditions (Rak et al., 2006) and in the spontaneously immortalized human RPE cell line ARPE-19 (Dunn et al., 1996), as well as in fetal cells (Gamm et al., 2008; Maminishkis et al., 2006). RPE65 and bestrophin proteins appear to accumulate only in cells of fetal origin (Gamm et al., 2008; Hu and Bok, 2001; Maminishkis et al., 2006). Melanization also appears to occur more reliably in cultured fetal RPE cells (Gamm et al., 2008; Maminishkis et al., 2006) than in human RPE cultures from adult donors where pigment accumulation is unpredictable, may require specific culture handling, and even then requires extended periods at confluency.

Perhaps even more important than cell-type-specific protein expression for gauging the maturity of RPE phenotype *in vitro* is determining whether the cultures display the appropriate sub-cellular localization of polarized proteins. The most common protein localization markers are adhesion proteins, especially AJ proteins N-cadherin and catenins (Burke et al., 1999; McKay et al., 1997; Rak et al., 2006), the TJ protein ZO-1 (Gamm et al., 2008; Geisen et al., 2006; Mandell et al., 2007; Rajasekaran et al., 1996; Tugizov et al., 1996), and junction-associated circumferential actin. The distribution of junctional proteins is typically analyzed by fluorescence microscopy in confluent cultures where the extent of epithelialization is estimated by inspecting the universality and completeness of a zonular pattern. The appearance of zonularity can be achieved for junctional proteins in most human RPE cultures, although, again, extended periods at confluency may be required and protein staining patterns are often heterogeneous among cells within the same cultures (Burke et al., 1996, 1999; McKay et al., 1997; McKay and Burke, 1994).

More critical evidence for RPE culture maturation is the attainment of a polarized protein distribution shown by morphologic methods such as confocal microscopy, or by the biochemical method of domain-specific cell surface biotinylation using cultures on filter supports that permit access to apical and basal surfaces. RPE polarity has been the topic of several reviews (Burke, 1998; Marmorstein et al., 1998; Marmorstein, 2001) in which many proteins are discussed that are preferentially enriched in either the apical or baso-lateral domains of mammalian RPE cells. The term ‘enriched’ is used because the protein asymmetry is not absolute. Further, polarization can result from a true asymmetry of the protein per unit area of membrane, or from different amounts of total membrane in apical versus basal domains (Marmorstein et al., 1998). The best known apically polarized protein of the RPE is Na/K ATPase which, as indicated above, is the opposite polarity found in most monolayer epithelial cells. Other apically enriched proteins in the RPE that were discussed in a review by Marmorstein (2001) include N-CAM (a cell adhesion molecule), EMMPRIN (an inducer of metalloproteinase secretion), αvβ5 integrin (a phagocytosis receptor), MCT1 (a monocarboxylate transporter), p75NTR (neurotrophic receptor), CFTR (cystic fibrosis transmembrane conductance regulator), and the *kir* family of K^+ channels. Additional apically polarized proteins in the RPE include the folate transport protein RFT-1 (Chancy et al., 2000), transport vesicle trafficking proteins syntaxin 1A and 1B (Low et al., 2002), and EBP50 (Bonilha and Rodriguez-Boulan, 2001), a binding partner of the actin-binding protein ezrin. Ezrin itself is also enriched in apical microvilli and used as an apical domain marker (Gamm et al., 2008; Kivela et al., 2000; Maminishkis et al., 2006). Some proteins shown to be baso-laterally enriched in the RPE are MCT3, the ezrin-binding protein SAP97, the folate receptor FRa, and bestrophin (Bonilha and Rodriguez-Boulan, 2001; Chancy et al., 2000; Marmorstein, 2001; Marmorstein and Kinnick, 2007).

Whether a specific polarized protein distribution in RPE cultures is considered ‘appropriate’ depends upon whether that distribution is known to occur in the tissue *in situ*. An apical polarity for Na/K ATPase has been demonstrated in the intact RPE of many mammalian (and non-mammalian) species (Bok, 1982; Burke et al., 2000; Gundersen et al., 1991; Rizzolo, 1990). However, for many of the proteins mentioned above, information on polarized distribution in mammals is limited to rodent RPE, especially rat. This species has been particularly useful for studies of protein polarization in the RPE because developmental stages *in situ* can be analyzed using embryos, and cultures are available for detailed analyses of polarization mechanisms, both primary RPE cultures and the immortalized rat line RPE-J (Nabi et al., 1993). There is a growing body of work using the rat model, much of it generated by Rodriguez-Boulan and his collaborators, devoted to identifying the mechanisms used by the RPE to sort proteins exhibiting a polarized distribution (Bonilha and Rodriguez-Boulan, 2001; Deora et al., 2004, 2005; Marmorstein et al., 1998; Marmorstein, 2001; Mora et al., 2006). However, relatively little is known about protein sorting mechanisms in human RPE, not only because the polarized

distribution of few proteins has been analyzed *in situ*, but also because human RPE cells, especially derived from adult donors, have a variable capacity to generate cultures that are highly polarized. As discussed further below, proteins that polarize variably or late in morphogenesis and therefore serve as benchmarks for a high epithelial maturation state (such as apical Na/K ATPase or basal bestrophin), usually fail to polarize in RPE cells from adult human donors.

Another category of markers for RPE maturation is functional measures of epithelial polarity such as transepithelial ion and nutrient transport, and the polarized secretion of growth and trophic factors. Such functions have been studied extensively in a variety of RPE preparations and cultures from several species including humans (Strauss, 2005). Regardless of what is transported or secreted, however, the ability of the RPE monolayer to vectorially move molecules is dependent upon the establishment of an effective barrier to paracellular flux. Barrier function, which defines a mature epithelium expressing tissue-level function, requires well-developed tight junctions and is commonly evaluated in cultures by measuring transepithelial electrical resistance across monolayers on filter supports. TJ formation in the pigment epithelium has been studied in some detail, especially in embryonic chick RPE investigated by Rizzolo and his collaborators (Rizzolo, 2007). It is clear from this body of work that TJ development is a complex progressive process during which there is sequential expression of genes for TJ proteins, an increase in the complexity of the tight junctional strands, a requirement for products of the neural retina *in situ* for junction maturation, and a dynamic remodeling and regulation of the TJ that determines its ongoing barrier function.

Indeed, TJ formation and other aspects of epithelial phenotype development are step-wise, time-dependent processes, giving cultured RPE cells the potential to exhibit a co-mingling of mature and immature traits.

3.2. RPE ‘re-morphogenesis’ in vitro

3.2.1. Cadherin cell–cell adhesion and RPE re-morphogenesis—As indicated above, the proteins comprising the epithelial adherens junction, especially E-cadherin, have been implicated as critical inducers of epithelial morphogenesis. However, E-cadherin is not the dominant cadherin of the RPE AJ, which is unusual for a monolayer epithelial cell type. For many years the RPE was believed to lack E-cadherin entirely (Gundersen et al., 1993; Huotari et al., 1995; Marrs et al., 1995), expressing instead B-, R-, P- and N-cadherin at various times during development and in various species (Huotari et al., 1995; Lagunowich and Grunwald, 1989, 1991; Liu et al., 1997; Murphy-Erdosh et al., 1994; Xu et al., 2002). For RPE cells in culture, including RPE cells from human donors (Burke et al., 1999; McKay et al., 1997; Rak et al., 2006; Van Aken et al., 2003), the dominant cadherin is N-cadherin.

As it turns out, E-cadherin is not completely absent from the RPE although it has a limited expression and junctional distribution. E-cadherin can be detected in extracts of human RPE cells, both from freshly isolated tissues and in primary cultures (Burke et al., 1999; Van Aken et al., 2003). However, the relative amount of E-cadherin protein, as compared to N- or P-cadherin, varies among samples from different donors (Burke et al., 1999). Further, E-cadherin localizes to junctions only in scattered patches of cells both *in situ* and *in vitro*, and long post-confluent time courses are required in cultured cells for E-cadherin to accumulate at junctional sites. Given the cell–cell heterogeneity in cadherin protein expression in RPE cells and the known morphogenetic function of E-cadherin, an obvious question to ask is whether RPE cell phenotype is related to cadherin phenotype. This question is another way of asking what was asked above: how much of what is known about E-cadherin-induced epithelial morphogenesis applies to the RPE. Do patches of cells with junctional E-cadherin differ phenotypically from cells that lack it? What role (if any) does N-cadherin, the dominant and ubiquitous cadherin of cultured RPE, play in RPE epithelial morphogenesis *in vitro*?

3.2.1.1. E-cadherin and RPE re-morphogenesis: In studies demonstrating the patchy accumulation of E-cadherin in the RPE (Burke et al., 1999) it was apparent that cultured cells do not have a homogeneous phenotype. Earlier investigations had shown striking morphologic heterogeneity among cells within the same human RPE cultures (Burke et al., 1996; Burke and Hjelmeland, 2005; McKay and Burke, 1994). Phenotypically distinct RPE subpopulations *in vitro* are revealed over a long post-confluent time course during which the cell morphologies diverge and, once established, remain stable for at least months provided that the phenotypes are not disrupted by dissociation and re-plating (Burke et al., 1996). RPE subpopulations with highly divergent morphologies, distinctly fusiform or highly epithelioid, can be partially separated by methods involving selective trypsinization of calcium-dependent adhesions (McKay and Burke, 1994). The effectiveness of this technique suggests that the complement of adhesive proteins, especially proteins associated with the calcium-dependent AJ, is likely to differ between RPE cell morpho types. However, in subsequent studies no differences were found between epithelioid and fusiform RPE in expression levels of several adhesion proteins (N-cadherin, α5β1 integrin, NCAM, PECAM) or other AJ-associated proteins that complex with cadherins or with cadherin-associated circumferential actin (α-catenin, β-catenin, plakoglobin, p120-catenin, α-actinin, vinculin) (McKay et al., 1997). The RPE phenotypic variants do, however, differ in junctional protein *localization*. Epithelioid but not fusiform cells undergo a slow, time-dependent protein re-distribution after confluency to generate a zonular organization of AJ proteins.

When E-cadherin was shown to accumulate slowly after confluency in the junctions of some human RPE cells, it was obvious to ask whether this known epithelial phenotype inducer was responsible for inducing the morphology of RPE subpopulations that appeared highly epithelioid. Examination of the relationship between junctional E-cadherin and RPE culture phenotype, however, did not resolve this issue and instead produced a more complex picture (Burke et al., 1999). RPE cells with junctional E-cadherin are, indeed, prominently epithelioid, but most clusters of epithelioid RPE lack junctional E-cadherin indicating that E-cadherin is not required for RPE cells to adopt a grossly epithelioid appearance. Rather E-cadherin accumulates in a subset of epithelioid cells late in confluency after the junction is formed. It then co-distributes with (but does not replace) N-cadherin.

Although E-cadherin is not required to produce highly epithelioid RPE, E-cadherin may nonetheless modify some aspects of RPE phenotype as indicated by distributional analyses of Na/K ATPase. As indicated previously, in other epithelial cell types E-cadherin engagement induces a baso-lateral distribution of the sodium pump (Marrs et al., 1993; Nelson et al., 1990). In cultured RPE cells Na/K ATPase usually fails to polarize, but when rat RPE cells were transfected to express high levels of exogenous E-cadherin, a baso-lateral sodium pump distribution was induced (Marrs et al., 1995). Given this observation, where is Na/K ATPase in epithelioid human RPE cultures and does its distribution differ in cells with, versus without, endogenous junctional E-cadherin? The answer to the latter is a qualified ‘yes’. In some highly epithelioid RPE cells Na/K ATPase polarizes in late confluency to the tissue-specific *apical* domain (McKay et al., 1997) (demonstrating, coincidentally, that some RPE cells from adult human donors are competent to achieve this marker of a high maturational state), but in the subset of epithelioid cells with junctional E-cadherin, the pump is non-polar (Burke et al., 1999). This outcome could result from E-cadherin having no effect on pump polarity, or conversely, E-cadherin could interfere with the apical polarization that can occur in epithelioid cells. The ‘interference’ theory is supported by observations on Na/K ATPase distribution *in situ* in bovine RPE (Burke et al., 2000). In bovine RPE, most cells show prominently apical Na/K ATPase as expected for this tissue, but similar to post-confluent cultures of human RPE, there are patches of cells scattered throughout the monolayer that have junctional E-(and/or P-)cadherin and in these patches the sodium pump protein is non-polar. Loss of apical pump polarity in E-/P-cadherin-positive RPE cells *in situ* appears to result from a smaller apical

microvillar domain, a taller lateral membrane domain, a basal enrichment of the ankyrin–fodrin membrane cytoskeleton with which the sodium pump associates in other epithelial cells, and more baso-lateral Na/K ATPase immunostaining. Although E-cadherin has not been expressly shown to *cause* this 'basal-ified' phenotype, it is consistent with E-cadherin induction shown in other epithelial cells.

If E-cadherin is responsible for the reduced apical pump polarity in E-cadherin-positive RPE cells *in situ* or *in vitro*, it is not clear why the baso-lateral induction is incomplete. One possibility that we previously suggested (Burke et al., 1999) is that E-cadherin's inductive capacity is reduced because the protein localizes to junctions late in their formation, after the N-cadherin adhesion is established. This possibility then raises the questions: why is junctional accumulation of E-cadherin delayed, and why does it localize to the junctions in only a subset of cells. These issues were considered in another investigation which showed that although the E-cadherin gene is constitutively expressed by human RPE cells, newly synthesized protein may be proteolytically processed to generate truncated peptides that transiently localize to organellar compartments rather than trafficking to membranes and inserting in junctions (Burke and Hong, 2006). Should small amounts of E-cadherin protein escape degradation, it could become enriched over time in the junctions of some cells accounting for the patchy junctional distribution of E-cadherin in post-confluent cultures.

Whether this theory proves to be accurate requires additional study. At this time it is not clear what function, if any, E-cadherin performs in RPE cells, either the truncated peptides found transiently in most cells or the junctional protein that accumulates in some epithelioid subpopulations. *In situ*, the patchy junctional E-cadherin in the RPE could simply be tolerated, an epi-phenomenon with little impact on tissue organization or function. Regardless, E-cadherin is largely absent from the RPE adherens junction in cultured cells in early confluency when the cells undergo morphogenesis. E-cadherin is therefore unlikely to play a major role in inducing RPE phenotype development.

3.2.1.2. N-cadherin and RPE re-morphogenesis: N-cadherin, not E-cadherin, is the most abundant cadherin expressed by RPE cells during re-morphogenesis *in vitro*. Given the pivotal role of cell–cell adhesion in epithelial morphogenesis, it is reasonable to ask whether N-cadherin participates in the induction of RPE epithelialization. Some years ago we examined the development of the zonular N-cadherin-based AJ in epithelioid RPE (McKay et al., 1997) and observed a process that resembled the process of E-cadherin junction formation in other monolayer epithelial cells. In both types of cells the cadherin undergoes a time-dependent localization to junctions, first distributing to puncta at cell–cell contact sites, then forming an incomplete or segmented adhesion that over time becomes progressively more zonular, more resistant to detergent extraction, and more tightly apposed to an increasingly compact circumferential actin band. However, there is a striking cell-type difference: the N-cadherin zonular junction requires weeks at confluency to develop in RPE cells, whereas the E-cadherin zonular adhesion develops in minutes to hours in other epithelial cell types. Coincident with the formation of the zonular AJ, both types of cells develop an epithelioid appearance, but while E-cadherin has been specifically implicated as an inducer of epithelial morphogenesis, N-cadherin has not.

The formed N-cadherin junction in RPE cultures is apparently competent to at least sustain epithelial morphology as indicated by the retention of an epithelial phenotype in post-confluent monolayers disrupted by wounding (Kaida et al., 2000). However, there is no explicit evidence to indicate that N-cadherin is required for RPE epithelial phenotype development, nor is there precedent to implicate N-cadherin as an epithelial morphoregulator. In fact quite the contrary, N-cadherin is typically found in non-epithelial cells. As indicated above (Section 2.2), when found in epithelial cells, N-cadherin is associated with disruption of an epithelial phenotype

and induction of a mesenchymal, migratory state – at least in carcinoma cells, which are the source of most information on N-cadherin in cells of epithelial origin due to its absence from most normal epithelial cells. There is some evidence, however, to suggest that N-cadherin interferes with, rather than generates, an epithelial cell shape in normal epithelial cells as well. In the normal kidney epithelial cell line MDCK, a major model of E-cadherin-induced morphogenesis, epithelial phenotype is disrupted in subpopulations of cells that express low levels of endogenous N-cadherin even on a backdrop of continued E-cadherin expression (Youn et al., 2005). This result is similar to the observation in carcinoma cells in which N-cadherin promoted motility regardless of the presence of E-cadherin (Nieman et al., 1999). In the RPE, too, N-cadherin has been implicated in inducing a fibroblastic and migratory or invasive morphology in pathologic states or models of pathologic states (Chen and Ma, 2007; Van Aken et al., 2003).

Given the evidence that N-cadherin in epithelial cells is usually associated with a mesenchymal phenotype, we previously theorized that RPE cells may develop an epithelial phenotype in spite of N-cadherin expression, rather than because of it (Youn et al., 2006). This suggestion was based partly on a study of an MDCK sub-clone that was selected for having a cadherin expression pattern resembling RPE cells: high endogenous N-cadherin and low E-cadherin. The MDCK sub-clone formed a zonular N-cadherin junction and, coincident in time, developed an epithelial phenotype, similar to RPE cells. However, as compared to morphogenesis in the E-cadherin-dominant parental MDCK line, junction formation and morphogenesis were slow in the N-cadherin-dominant sub-clone, again similar to RPE cells. This observation of delayed morphogenesis when N-cadherin dominates raises the possibility that N-cadherin may be compatible with the development of a zonular AJ in normal epithelial cells, but it may not, like E-cadherin, facilitate it.

If N-cadherin does not drive the development of an epithelial-type AJ in normal epithelial cells, why do cultured RPE cells form an AJ that is zonular? In non-epithelial cells like neurons, cardiac muscle or fibroblasts where N-cadherin is the typical dominant cadherin (Hatta and Takeichi, 1986; Kaufmann et al., 1999; Matsuyoshi and Imamura, 1997; Pouliot et al., 1990), N-cadherin forms a variety of cell-type-specific adhesion plaques suggesting that N-cadherin AJ morphology is determined by cellular context. In normal epithelial cells like the RPE an epithelial cell shape may therefore arise by a mechanism that is not directly related to cadherin induction. One attractive possibility is that RPE morphogenesis results from actin re-organization driven by mechanisms that induce the dominant actin cytoskeleton to remodel from the linear arrays seen in subconfluent cells to the circumferential band that characterizes an epithelial phenotype at confluency (Pletjushkina et al., 1994; Yonemura et al., 1995). According to this view, a circumferential actin pattern would develop in normal epithelial cells regardless of cadherin type, although different cadherins could differentially affect the zonular actin bundle; E-cadherin may accelerate its formation, while N-cadherin may passively associate with it after it is formed. This possibility implies a role for actin rearrangements *upstream* of cadherin engagement in the process of epithelialization, which is consistent with data implicating actin remodeling as a key step in epithelial morphogenesis (Perez-Moreno and Fuchs, 2006).

There is currently no evidence implicating specific actin remodeling pathways in epithelial cells like the RPE that produce a zonular AJ containing N-cadherin, although there are some tantalizing observations to suggest that actin rearrangements could differ with cadherin type. At the forming E-cadherin AJ, p120-catenin plays an important role in orchestrating actin dynamics thereby integrating cytoskeletal rearrangements, junction formation and morphogenesis (Perez-Moreno and Fuchs, 2006). However, p120-catenin is known to interact differently with E- and N-cadherin, associating at different times during cadherin–catenin complex assembly (Davis et al., 2003; Miranda et al., 2003; Wahl et al., 2003), and performing

different functions in cadherin trafficking and retention at surface membranes (Chen et al., 2003; Davis et al., 2003). When expressed within the same epithelial cells, p120-catenin also forms stoichiometrically different complexes with E- and N-cadherin (Youn et al., 2005).

Whether p120-catenin regulates actin dynamics during the development of a zonular N-cadherin AJ in RPE cells or mediates other aspects of RPE epithelial morphogenesis has not been investigated. In general, the molecular mechanisms of N-cadherin AJ formation are poorly understood, but generating a zonular organization of this structure is likely to be critical for morphogenesis of the RPE. The earliest events in the formation of an E-cadherin epithelial AJ in other cells, those associated with generating nectin-based adhesions and associated actin remodeling, are unexplored in the RPE. Investigations patterned on studies of the E-cadherin AJ, especially with regards to cytoskeletal dynamics, could be particularly revealing for the ascertaining the role of cadherins in RPE morphogenesis. Further, the impact of this approach might be much broader. From the point of view of cancer biology, the RPE can be viewed as having a cadherin expression pattern, with its high N-cadherin and low E-cadherin, that simulates a metastatic carcinoma. Yet the RPE is able to develop a non-motile epithelioid morphology in culture, and metastatic tumors of the RPE *in situ* are exceedingly rare (Loeffler et al., 1996). Understanding how RPE cells develop and sustain an epithelial phenotype with N-cadherin as their dominant AJ protein therefore could not only aid in the development of strategies to maximize RPE re-morphogenesis and therefore tissue repair, but also have widespread implications for understanding dysregulation of adhesions in cancer.

3.2.2. RPE re-morphogenesis and re-pigmentation—As indicated in the previous sections, a mature phenotype in monolayer epithelial cells is characterized by cytoarchitectural features determined in large part by the development of cell–cell adhesions; for the RPE another important indicator of phenotypic maturity is pigmentation.

RPE cells synthesize melanin *in situ* during embryogenesis, after which pigment turnover is believed to be quite low (Boulton, 1998; Schraermeyer and Heimann, 1999). When RPE cells are propagated in culture they lose pigmentation as pigment granules (melanosomes) are exocytosed or diluted by cell division. RPE cells cultured from adult human donors typically remain amelanotic after pigmentation is lost because, despite expression of the genes required for pigment synthesis such as tyrosinase and tyrosinase-related proteins (Tyrps) (Abe et al., 1996, 1998; Abul-Hassan et al., 2000), the proteins apparently fail to accumulate. Nonetheless, several investigators have reported re-melanization of human RPE cultures, either ‘spontaneously’ or because of some combination of medium additives, culture substrate modification or culture handling (Campochiaro and Hackett, 1993; Dorey et al., 1990; Gamm et al., 2008; Maminishkis et al., 2006; Ohno-Matsui et al., 2005; Pfeffer et al., 1986; Rak et al., 2006; Song and Lui, 1990). Like epithelial phenotype development, melanin biosynthesis, when it occurs, is a protracted process, often requiring weeks at confluency for pigment to accumulate (Fig. 1).

Cultures from fetal human donors appear more likely to re-pigment than RPE cells from other sources. These cells also achieve the highest overall morphogenetic state observed for cells propagated from the RPE. Highly developed fetal RPE cultures were described some years ago (Hu and Bok, 2001) and more recently protocols were developed that produced cultures in which prominent pigmentation was described as an explicit feature (Gamm et al., 2008; Maminishkis et al., 2006). In addition to pigmentation, the cultures exhibited an array of markers of high epithelial maturation including gene and protein expression of RPE65 and CRALBP, abundant apical ezrin, and (notably) expression and basal polarization of bestrophin (Gamm et al., 2008). It should be noted that fetal RPE are not typically passaged by enzyme treatment to dissociate cells, the protocol commonly used to propagate cultures from post-natal

donors. As the discussion below implies, limiting degradation of cell adhesions could be a significant contributor to the higher level of morphogenesis attained by fetal cells.

An interrelationship between heavy pigmentation, a striking epithelial phenotype and cell–cell adhesion in RPE cultures was illustrated by the McKay laboratory (Rak et al., 2006). Using RPE cultures from adult human donors and a protocol that did not require specialized culture medium or coated substrates, this group was able to trigger a reversible shift in RPE culture phenotype from fusiform with no pigmentation to epithelioid with pigmentation. The method employed a calcium switch, which demonstrates an intriguing link between calcium-dependent adhesions and morphogenesis. According to the protocol, cells are initially plated at high density in culture medium containing calcium levels that are too low to support cadherin-dependent adhesion. Then days later, when cultures are switched to medium containing its normal high calcium content, adhesions rapidly form from cadherins pre-existing in membranes. The calcium switch protocol was developed several years ago to synchronize the timing of assembly of E-cadherin adhesions in MDCK epithelial cells (Nelson and Veshnock, 1987). In RPE cells, the switch is followed by N-cadherin adhesion formation and then days to weeks later by the expression of RPE maturation marker proteins (e.g., tyrosinase and CRALBP) and ultimately by the generation of phenotypically epithelioid and pigmented monolayers (Rak et al., 2006).

What underlying mechanisms could account for the effectiveness of the calcium switch protocol in triggering both RPE epithelialization and melanization? Given the long time interval between the trigger and the ultimate outcome, a complex cascade of mechanisms is likely induced that involves transcription factor networks and multiple signaling pathways. Sorting these out in RPE cells will be a challenge, but guidance might be provided by analyzing components of the Wnt/β-catenin signaling pathway.

4. Convergence of adhesion, morphogenesis and pigmentation: the Wnt/β-catenin pathway

The Wnt signaling pathway plays a pivotal role in regulating tissue differentiation not only during embryogenesis, but also post-natally where it functions in stem cell renewal, cancer and even aging (Clevers, 2006; Daugherty and Gottardi, 2007; Gavert and Ben-Ze'ev, 2007; Meshorer and Gruenbaum, 2008; Nelson and Nusse, 2004; Polakis, 2007). A central mediator of the canonical Wnt pathway is β-catenin, which, as indicated above, also binds the cytoplasmic domain of cadherins, indicating a point of convergence between the Wnt pathway and the cadherin adhesion system (Nelson and Nusse, 2004).

In the absence of Wnt signaling, β-catenin is complexed in the cytoplasm with other proteins including axin, adenomatous polyposis coli (APC) protein, and glycogen synthase kinase-3β (GSK-3β), which phosphorylates proteins in the complex and targets β-catenin for rapid degradation (Fig. 2). The Wnt pathway is activated by extracellular Wnt ligands that bind cell surface receptors (frizzleds) and co-receptors (lipoprotein receptor-related proteins 5/6 [Lrp5/6]) activating a signaling pathway that inhibits GSK-3β activity leading to the reduced degradation of β-catenin. β-Catenin then accumulates in the cytoplasm and translocates to the nucleus where it binds to the T cell factor (TCF)/lymphoid-enhancing factor (LEF) transcription factor machinery and promotes activation of target genes, notably proliferation regulators such as cyclin D1 and c-myc (Clevers, 2006;Gavert and Ben-Ze'ev, 2007;Thompson and Monga, 2007). β-Catenin pool size in the cytoplasm and thus its availability to participate in signaling is also regulated by cadherin binding (Nelson and Nusse, 2004).

Consideration of a role for the Wnt/β-catenin pathway in RPE morphogenesis is motivated by several considerations. Some are related to the functions that have been identified for the

pathway in affecting morphogenesis of other epithelial cells, and others relate specifically to the RPE.

From the point of view of epithelial morphogenesis, activation of the Wnt/β-catenin pathway is known to participate in the loss of epithelial phenotype that accompanies epithelial–mesenchymal transitions (EMT) during embryogenesis and cancer progression, or when induced experimentally in normal epithelial cell lines (Doble and Woodgett, 2007; Katoh and Katoh, 2006; Moustakas and Heldin, 2007). Central to EMT is changes in the phosphorylation state of the pivotal Wnt mediator β-catenin and, upstream of β-catenin, GSK-3β. GSK-3β is also located at the intersection of Wnt/β-catenin pathways and pathways induced by FGF and TGFβ, which change the expression or activation state of their transcriptional regulators Snail and Smads, thereby acting synergistically to affect EMT (Katoh and Katoh, 2006; Moustakas and Heldin, 2007; Nelson and Nusse, 2004).

Aside from its role in regulating phenotype in other epithelial cells, there are specific reasons to consider Wnt/β-catenin signaling as an important pathway in RPE morphogenesis. A direct target gene of the Wnt/β-catenin pathway is the Microphthalmia-associated transcription factor (Mitf), a key regulator of the formation of pigment cells, including the RPE (Deora et al., 2005; Larue and Delmas, 2006; Murisier and Beermann, 2006; Shibahara et al., 2000, 2001; Steingrimsson et al., 2004; Tachibana, 2000). Mitfs transactivate promoters of the tyrosinase gene family to induce expression of tyrosinase and tyrosinase-related proteins-1 and -2 thereby controlling pigment synthesis (Fig. 2). Mitf was also recently shown to positively regulate expression of the VMD2 gene that encodes bestrophin (Esumi et al., 2007), described above as a significant marker of RPE maturation. A role for Wnt signaling in RPE morphogenesis is further indicated by the observation that certain mutations in the APC gene produce a discrete RPE phenotype. As indicated above, the APC protein is a component of the degradation complex that regulates β-catenin availability for Wnt signaling. APC mutations produce familial adenomatous polyposis (FAP), a syndrome that predisposes to colorectal cancer (Ballhausen, 2000). Some variants of FAP result in congenital hypertrophy of the RPE (CHRPE), which is characterized by nodular RPE lesions that are often highly pigmented (Tiret and Parc, 1999).

Fragmentary evidence therefore suggests that investigating the Wnt/β-catenin pathway in RPE cells could provide a useful framework for probing mechanisms of RPE morphogenesis. However, determining whether/how this signaling network functions in the RPE is not trivial. Returning to the calcium switch protocol that induced both epithelialization and pigmentation in cultured human RPE (Rak et al., 2006), one potential result of the switch that implicates the Wnt pathway would be an abrupt shift in the balance of β-catenin available for signaling. As cadherin adhesions rapidly form in high calcium, β-catenin is theoretically stabilized at junctions reducing the signaling pool and down regulating the expression of Wnt target genes (Nelson and Nusse, 2004). A transient change in the activity of signaling pathways activated on cadherin engagement (including Wnt) can then have consequences that play out over long time frames, which appears to be the case for RPE morphogenesis triggered by the calcium switch. Changed gene expression patterns may be sustained and/or may induce a cascade of signaling events, perhaps including recurrent Wnt signaling episodes. The latter is suggested because the reduced expression of TCF/LEF target genes resulting from reduced Wnt signaling theoretically produces some outcomes that enhance and others that reduce RPE phenotype development. An example of the former might be reduced proliferation, which is permissive for epithelial maturation, and an example of the latter is decreased expression of Mitf, which is required for pigmentation and bestrophin expression.

A complex Wnt output is expected since Wnt signaling activates endogenous transcriptional cascades that vary with cell type and cell state. Cell proliferation, for example, is promoted

under some conditions by Wnt signaling and terminal differentiation under others (Clevers, 2006). For the RPE, Wnt signaling may be required during embryogenesis for tissue specification and pigmentation, but if the RPE behaves like other epithelial cells, re-activation of Wnt signaling in the mature tissue may induce EMT. The ultimate effect of a change in the activity of the Wnt pathway is therefore determined by cellular 'background'. An important component of that background is the expression of receptors and the availability of ligands that activate Wnt/β-catenin intersecting pathways. As implied above, several pathways converge on GSK-3β. Further, β-catenin itself is regulated by phosphorylation, not only in the degradation complex of the Wnt pathway but also by receptor and cytoplasmic tyrosine kinases in the cadherin–catenin complex at adhesion sites (Daugherty and Gottardi, 2007; Nelson and Nusse, 2004). The signaling background produces complex feedback loops that modify the output of the Wnt signal. For example, in addition to being a target gene of TCF/LEF transcription factors, the Mitf protein can directly bind β-catenin in a feedback mechanism that may regulate Wnt/β-catenin signaling (Schepsky et al., 2006). Further, Mitf in some pigmented cells is negatively regulated by FGF and positively regulated by TGF-β via their induced transcription factors such as SMADs (Bharti et al., 2006).

As implied above, the morphogenetic response of RPE cells to the calcium switch is apparently not due to a single event but rather due to the activation of a morphogenetic cascade. Further, cultures differ in their competence to respond to the switch, and in those cultures that do respond the re-pigmentation starts in scattered cells in the culture monolayer and subsequently spreads outward to neighboring cells (Rak et al., 2006). This intriguing observation suggests that at some critical stage in development, RPE cells may produce mediators that stimulate immediately adjacent cells, perhaps by gap junctional communication and/or by a paracrine mechanism that involves short-lived or low abundance mediators (perhaps Wnts?). Regardless of the mechanism, the observation that a subset of cells responds first and adjacent cells respond later implies that individual cells vary in their background active signaling networks, and that the triggers for morphogenesis are sequential.

Despite the complexity of the signaling networks that underlie RPE morphogenesis, sorting them out is essential to increase our understanding of RPE phenotype development and retention. There are currently few investigations of the Wnt pathway in cultured RPE cells that might inform us about the pathway's role in epithelialization. Inoue et al. (2007) recently analyzed the conditioned medium from rat RPE cultures using a reporter system with a TCF responsive element and identified an activity secreted by the RPE that suppresses Wnt signaling. This work, which followed studies implicating Wnt pathways in the rd6 mouse (Jones et al., 2000; Kameya et al., 2002; Mandal et al., 2006), demonstrated a potential for the RPE to produce Wnt effectors. Cells cultured from embryonic chick RPE have been used to implicate Wnt pathways in RPE cell differentiation/transdifferentiation, primarily via expression and function of Mitf (Iwakiri et al., 2005; Mochii et al., 1998). Of particular relevance to the issue of epithelial phenotype development *in vitro*, suppression of Mitf by small interfering RNA induced de-differentiation of chick RPE, which was indicated by a reduction in a pigmentation marker (melanosome glycoprotein 115) and by a loss of gross epithelial phenotype (Iwakiri et al., 2005). The latter manifested as a shift in the organization of the dominant actin cytoskeleton from a zonular band, typical of epithelial cells, to linear stress fibers, typical of mesenchymal cells.

For human RPE, there is only scattered evidence to indicate that known or potential effectors of the Wnt pathway are expressed and even less is known about Wnt signaling in cultured cells that could implicate a role for this pathway in morphogenesis. Expression of genes for the Wnt receptor FZD7 (Katoh and Katoh, 2007) and for a modulator of Wnt ligand binding SFRP 5 (secreted frizzled-related protein 5) (Chang et al., 1999) have been detected in human RPE (although the latter was no longer expressed when cells were cultured (Chang et al., 1999).

Membrane frizzled-related protein (MFRP), which has a Wnt binding motif, was found in human RPE-choroid extracts (Mandal et al., 2006). (There is an ocular phenotype associated with MFRP mutations, which cause extreme hyperopia or nanophthalmos in humans (Sundin et al., 2005) and produce the progressive retinal degeneration found in the rd6 mouse (Kameya et al., 2002).) Explicit studies of Wnt pathways in human RPE cultures are few, with only one recent study probing the Wnt pathway in human RPE cells in the context morphogenesis (Krugluger et al., 2007). Even this investigation, which used the ARPE-19 cell line, was somewhat indirect, focusing not so much on Wnt signaling but rather on transcriptional regulation of Wnt proteins (β-catenin and GSK-3β) and markers of RPE differentiation (cytokeratin 18 and RPE65) by treatment of cells with ligands for receptor tyrosine kinases (e.g., EGF).

It is probably fair to say that despite the known role of Wnt signaling in morphogenesis of other epithelial cells and in RPE specification during embryogenesis, this pathway is virtually unexplored as a regulator of cell phenotype in cultures of the RPE.

5. RPE ‘de-morphogenesis’ and aging

5.1. RPE phenotype: pathology and aging

Research on RPE morphogenesis is partly driven by a basic interest in determining how cells acquire their cell-type-specific phenotype. Research is also strongly motivated by a desire to understand and ultimately control RPE phenotype in pathologic states. An area of current interest in RPE morphogenesis comes from the field of RPE transplantation. Transplantation is being explored as a mechanism to treat age-related macular degeneration (AMD) by replacing the aging, dysfunctional RPE with healthy tissue that is better able to support retinal photoreceptors. One strategy for generating a graft is to amplify RPE cells by culturing them. However, this approach has been stymied by the problems discussed above: RPE cells lose their phenotype on *in vitro* propagation, and then fail to fully re-epithelialize after confluency is attained. (The reader is referred to a recent review that thoroughly covers the topic of RPE transplantation in AMD (Binder et al., 2007). In that review, RPE phenotype is also discussed in some detail, including topics significant for RPE morphogenesis that are not discussed here. Notable among these are the effects of substrate and of humoral agents, including factors produced by the RPE itself, on cell phenotype.)

A presumption underlying efforts to transplant RPE to restore function to the aging monolayer is that RPE cells lose their normal, functionally optimal phenotype as they age. Although aging of the RPE has been the topic of many investigations, few relate specifically to whether aging affects the organizational state of the RPE as a polarized monolayer epithelium. Rather studies of aging of the RPE *in situ* have focused on changes in granule content, and on topographical differences between the macula and periphery that might explain why the macula is predisposed to age-related degeneration (Boulton and Dayhaw-Barker, 2001). The granule content of RPE cells clearly changes with age; unmodified melanin granules decline while lipofuscin and complex granules (melanolysosomes and melanolipofuscin) increase. There is also considerable evidence to indicate that macular and more peripheral RPE cells differ, both throughout life and as a consequence of age, in several fundamental properties including granule content, the expression of certain genes or proteins, and the activity of various enzymes (Burke and Hjelmeland, 2005; Kociok and Joussen, 2007; Mullins et al., 2007).

With regards to the specific issue of RPE cell phenotype and aging, the most relevant investigations have to do with age-related RPE cell loss, which would inevitably produce some organizational remodeling of remaining cells. RPE cell loss appears to occur with age, although the extent and topography are not entirely clear (Boulton and Dayhaw-Barker, 2001; Del Priore et al., 2002; Dorey et al., 1989; Feeney-Burns et al., 1990; Gao and Hollyfield, 1992; Watzke

et al., 1993). One investigation with particular relevance for RPE phenotype was the work of Watzke et al. (1993) that demonstrated an age-related diminution in the hexagonality of foveal RPE. Decreased regularity in RPE cell shape can be viewed as a gross phenotypic manifestation of a reduced organizational state of the cells; however, whether and how aging might change the specific markers of epithelial maturation and polarity discussed above is undetermined.

Indeed, we have no standardized mechanism for defining alterations of RPE morphology *in situ* (Mecklenburg and Schraermeyer, 2007) and aging changes in RPE epithelial organization, should they occur, are likely to develop slowly and perhaps be quite subtle. Age-related structural changes may also be highly heterogeneous, not only across broad topographical regions (macula vs. periphery, for example), but also focally. The RPE monolayer is a mosaic of structurally variable cells regardless of age (Burke and Hjelmeland, 2005). Identifying aging changes in RPE cell phenotype on this variable background may therefore require detecting whether aging changes the *frequency* of cells that exhibit specific features of a mature polarized epithelium. Given donor variability, RPE cell–cell heterogeneity, and the complexity of aging plus its long time line, identifying phenotypic changes associated with normal aging could be a daunting task.

Although specific evidence for an effect of age on RPE phenotype *in situ* is limited, examination of cultured cells supports the notion that age affects cell organization. RPE cultures from adult human donors are typically used in early passage where they are presumed to display features most like the normal tissue. With repeated passage to induce senescence by forced replication, cultured RPE cells from both animal and human eyes become progressively more heteromorphic (Burke and McKay, 1993; Hjelmeland et al., 1999; Lee et al., 2001). Indeed, a morphology that deviates from the epithelioid – what could be called ‘de-morphogenesis’ – is a hallmark of aged RPE cultures.

5.2. Age-related RPE ‘de-morphogenesis’: oxidative stress and the Wnt/β-catenin pathway

How could aging *in situ* produce changes in the architecture of cells in a long-lived post-mitotic tissue like the RPE? One attractive possibility is that phenotype could be affected as a consequence of age-related oxidative stress.

The topic of oxidative stress and the RPE has a significant history and a growing recent interest. The focus in this field has been on the role of stress to the RPE as a contributing factor to AMD (Algvere et al., 2006; Bazan, 2008; Cai et al., 2000; Liang and Godley, 2003; Wu et al., 2006; Zarbin, 2004). The identification a few years ago of genes that confer AMD risk had a major impact on AMD research (Edwards et al., 2005; Hageman et al., 2005; Haines et al., 2005; Klein et al., 2005), but how aging itself contributes to disease onset, progression and severity remain important questions, and oxidative stress has long been recognized as a major aging influence (Simm et al., 2008). AMD is believed to originate in the RPE where the earliest manifestations of the disease are seen, and oxidative stress has been implicated partly because the tissue is at unusually high risk for oxidative injury due to its location and function. The RPE resides in a highly oxygenated environment adjacent to the capillary bed of the choroid, and the tissue is exposed throughout life to light, including highly phototoxic blue light that produces cellular damage by oxidative mechanisms (Algvere et al., 2006; Wu et al., 2006). The RPE must also cope with a daily load of membranes that are rich in polyunsaturated fatty acids (Stinson et al., 1991) due to its function of phagocytizing shed photoreceptor outer segments. Although it is difficult to prove a direct link between oxidative stress and any slow-to-develop disease of aging, a role for stress in AMD is inferred from studies indicating that antioxidant supplementation has a protective effect (AREDS, 2001; Tan et al., 2008), and that life style factors with an oxidative stress component (such as cigarette smoking) confer disease risk (Klein et al., 2008; Zarbin, 2004).

There are many studies on the RPE using cultured cells that involve inducing oxidative stress with chemical oxidants or light irradiation. These treatments are followed by analyses of stress pathways, or by investigations of agents that exacerbate (prooxidants) or protect against oxidative damage (antioxidants and trophic factors) (Algvere et al., 2006; Bazan, 2008; Cai et al., 2000; Wong et al., 2007; Wu et al., 2006; Zarbin, 2004). The primary outcomes in most such studies are measures of cell death after induction of lethal stress, an experimental strategy that is based on the likelihood that oxidative stress contributes to AMD by inducing RPE cell loss via apoptosis (Cai et al., 2000; Del Priore et al., 2002; Dunaief et al., 2002).

However, if the issue is not the role of oxidative stress in RPE *pathology* but rather in *normal aging*, the relevant oxidative injury is not lethal but sub-lethal. Stress associated with aging results in a gradual loss in functional efficiency that only over time becomes sufficient to cause significant impairment or cell death. Considering the effects of sub-lethal stress on the RPE, there is strong rationale for theorizing that an important consequence would be 'de-morphogenesis' or a loss of cellular organization. Among the most highly susceptible biomolecules to oxidative modification are cytoskeletal proteins, notably actin and tubulin (Aslan et al., 2003; Banan et al., 2001, 2003, 2004; Dalle-Donne et al., 2001a, 2001b, 2002; Fratelli et al., 2003; Keshavarzian et al., 2003). These proteins comprise the interacting microfilament and microtubular networks that are important determinants of cell shape. As discussed above, regulated cytoskeletal rearrangements, especially actin dynamics, contribute to the generation and maintenance of an epithelial phenotype. However, cytoskeletal organization is sensitive to oxidative stress in many epithelial cell types, including in the RPE (Akeo et al., 2002; Bailey et al., 2004; Garg and Chang, 2003)(Fig. 3).

Indeed, in cultured RPE cells several markers of RPE epithelial maturation are susceptible to sub-lethal stress (Bailey et al., 2004; Ho et al., 2006). In addition to inducing re-organization of the actin cytoskeleton from circumferential to stress fiber-like, treatment of confluent ARPE-19 cells with the oxidant H_2O_2 also causes a decline in barrier function of the monolayers, and a loss of junctional integrity indicated by reduced association with junctional sites of both TJ (ZO-1; occludin) and AJ (N-cadherin and β-catenin) proteins. The latter changes occur in vivo as well as demonstrated in mice deficient in a major anti-oxidant system (SOD-1) where induction of oxidative stress produces a loss of junctional N-cadherin and β-catenin in the RPE (Imamura et al., 2006).

Accompanying the displacement of β-catenin from junctions by H_2O_2 treatment of ARPE-19 cells is an increase of β-catenin in the cytosol, which could increase its availability for signaling (Bailey et al., 2004). This observation is particularly interesting in the light of recent reports describing another important convergent pathway involving the Wnt mediator β-catenin. In addition to linking adhesion, morphogenesis and pigmentation via TCF/LEF transcriptional activation as discussed above, the Wnt/β-catenin pathway also links oxidative stress and aging (Bowerman, 2005; Essers et al., 2005; Liu et al., 2007; Manolagas and Almeida, 2007; White et al., 2007) (Fig. 2). β-Catenin can protect cells against oxidative stress by acting as a cofactor for another class of transcription factors, forkhead box O (FOXO) factors, which are induced by reactive oxygen species and which regulate expression of genes for antioxidant enzymes and for proteins involved in DNA repair (Calnan and Brunet, 2008). FOXOs not only protect against oxidative stress, but also regulate lifespan in animal models of longevity. FOXOs and TCF/LEF transcription factors may compete for β-catenin, thereby producing complex antagonisms between the two pathways. With the exception of the observation that homogenates of the RPE from donors with AMD showed upregulation of some FOXO target gene products (Decanini et al., 2007), little is known about whether the Wnt mediator β-catenin participates in pathways regulating the RPE response to oxidative stress or cell longevity.

The mechanisms whereby sub-lethal oxidative stress to the aging RPE could dysregulate cell phenotype are virtually unexplored. In other epithelial or endothelial cells where this question has been addressed, changes in turnover of polarized proteins (Grune et al., 2002) and in many signaling pathways, notably those regulating adhesions (Basuroy et al., 2003; Katsube et al., 2007; Krizbai et al., 2005; Rao et al., 2002; Saenz-Morales et al., 2006; Usatyuk et al., 2006), have been implicated. Cytoskeletal remodeling is a near universal observation in oxidatively stressed cells, but the exact mechanisms whereby this occurs remain to be determined. Alterations in the activity of actin effectors Rac1 and RhoA have been reported (Saenz-Morales et al., 2006), but undoubtedly multiple additional mechanisms are involved as well.

Whatever the mechanisms of stress-induced cytoskeletal alterations, widespread secondary effects on cell phenotype would likely result since the cytoskeleton is critical to overall cytoplasmic organization. There is a growing body of evidence to indicate that actin microfilaments, microtubules and their associated motors are required for the trafficking of membranes, organelles, and messenger RNAs to the cytoplasmic compartments where they function (Boldogh and Pon, 2007; Kloc and Bilinski, 2003; Lopez and Jansen, 2004; Stebbings, 2001; Tekotte and Davis, 2002). Even ‘soluble’ molecules in the cytoplasm are enriched in cellular sub-domains by the geometry of the cytoskeletal network (Leterrier, 2001). All cells translocate their organelles such as mitochondria (Boldogh and Pon, 2007) using cytoskeletal scaffolds, and for pigmented cells like the RPE, cytoskeletal networks and their motors are also essential for melanosome positioning (Barral and Seabra, 2004; Coudrier, 2007; Futter, 2006). Indeed, melanosomes have been an important model for studying actin-based and microtubule-based organelle motility (Barral and Seabra, 2004). Of specific relevance for epithelial phenotype, trafficking along cytoskeletal scaffolds, especially of endosomes, is required for epithelial cell polarity (Apodaca, 2001; Rodriguez-Boulan et al., 2005), including polarity of the RPE (Rodriguez-Boulan et al., 2005). If sub-lethal oxidative stress disrupts cytoskeletal organization in RPE cells as the available evidence suggests, inappropriate positioning of cellular components – a reasonable definition of loss of cell phenotype – seems unavoidable.

We are currently conducting investigations to determine whether sub-lethal oxidative stress impairs a trafficking function of cultured RPE cells, namely organelle translocation. Melanosomes are used for these studies not only because they are relevant for the RPE, but also because the granules can be effectively tracked by light microscopy in living cells. In these investigations melanosomes are isolated from porcine RPE then delivered to ARPE-19 cells for uptake by phagocytosis using low granule numbers to produce limited overlap of the trajectories of individual granules. Blue light illumination of the cells is used to induce oxidative stress (Algvere et al., 2006; Wu et al., 2006) and cells are imaged before and after light treatment to track granule motility (Fig. 4). Cells are also imaged for several additional hours after light exposure to confirm cell survival. In experiments to be reported in detail elsewhere, granule movement is observed to slow significantly when RPE cells are subjected to sub-lethal photic stress (Fig. 4). This observation is probably not specific for melanosomes but may apply to other membrane-bound organelles as well. Indeed the melanosomes used here should be more precisely considered phagosomes since the granules were internalized by phagocytosis; melanosomes and phagosomes move by similar mechanisms (Algvere et al., 2006; Barral and Seabra, 2004; Futter, 2006; Seabra and Coudrier, 2004). It appears from these studies that sub-lethal oxidative stress to the RPE can reduce the efficiency of organelle translocation. This raises the possibility that repeated or chronic low levels of stress associated with aging could affect RPE phenotype *in situ* by leading to a gradual mis-localization of organelles. Perhaps this mechanism accounts for the observation that melanosomes of the aging RPE are less apically restricted than in young cells (Feeney, 1978). Further, a stress-induced slowing of organelle movement may have ramifications for the efficiency of RPE cells to translocate phagosomes containing photoreceptor outer segments from the cell surface to the

peri-nuclear region where they fuse with lysosomes to degrade phagosome contents. Stress-induced organelle slowing would not only affect organelle distribution, but also theoretically impact photoreceptor survival by impairing the essential process of photoreceptor turnover.

There is no specific evidence at this time to indicate that the stress-induced organelle slowing we have observed results from oxidative modification of the RPE cytoskeleton *per se.* Indeed, the sub-lethal stress levels that are used do not overtly disrupt the gross actin or microtubule networks within cells that are detectable by light microscopy (see sub-lethal photic treatment in Fig. 3). Many other mechanisms can be envisioned that could affect motility, ranging from dysregulation of motor complexes to insufficient energy. However, it seems safe to say that sub-lethal oxidative stress to the RPE over time has the potential to produce widespread derangements in sub-cellular organization. Effects of these changes on cell morphology with aging may be subtle, but RPE cell ‘phenotype’ is more than the superficial microscopic picture of the tissue. It is the cell's ‘molecular phenotype’ that ultimately determines the functional efficiency of the RPE, but our understanding of this level of cellular organization is still in its infancy.

6. Summary and future directions

RPE cells present an interesting challenge for students of epithelial morphogenesis. In the tissue *in situ*, RPE cells are growth quiescent with a highly regular cell shape. The cells can remain in this state in the human eye for decades, and yet retain the capacity to de-epithelialize and to grow when explanted into cell cultures. However, for reasons that remain elusive, after propagation *in vitro*, the cells show an incomplete and variable ability to re-develop an epithelial phenotype. In recent years cultured RPE cells have proved quite useful for exploring cell-type-specific pathways to establish protein polarity, pathways that are central to epithelial morphogenesis. However, initial events in RPE phenotype development, events that first demarcate the apical and basal membrane domains and that are a prerequisite for polarization are still poorly understood.

In other epithelial cells, formation of an E-cadherin intercellular adhesion plays an important early inductive role in morphogenesis. However, this protein is largely absent from RPE cells during *in vitro* phenotype development and it is not clear whether or how the dominant RPE cadherin, N-cadherin, participates in morphogenesis. Recent studies of the E-cadherin-containing adherens junction in other epithelial cells point to a critical role in phenotype development for actin cytoskeletal remodeling at the AJ. Actin rearrangements also accompany the formation of nectin-based adhesions that precede AJ assembly and the development of the tight junctions that follow. The ultimate outcome is an actin organization that is dominantly zonular, a fundamental epithelial characteristic. In RPE cells, analyses of actin dynamics during the maturation of N-cadherin adhesions, patterned on the recent studies of E-cadherin in other epithelial cells, may be a fruitful strategy for determining how RPE cells generate an epithelial-type zonular AJ. Regarding actin dynamics, analysis of the functions of the cadherin-binding regulatory protein p120-catenin may be a useful place to start. It is probably also time to ask whether RPE cells express additional newly identified or minor cadherins that could participate in junction assembly or phenotype induction.

Aside from failure to regenerate a highly epithelioid cell shape, another phenotypic deficiency of cultured human RPE is the absence of pigmentation. An argument is made here that phenotype development and melanization are linked, and that both might be regulated by a common signaling pathway: the Wnt/β-catenin pathway. Currently, evidence implicating this widespread and highly complex pathway in RPE morphogenesis is suggestive at best. Nonetheless, the pathway could provide a useful framework for teasing out the steps in RPE morphogenesis, which appears to be a slow, step-wise process with many points of regulation

where cell maturation can cease, or perhaps be diverted. A judicious and methodical series of investigations of the Wnt/β-catenin pathway in RPE cells will not be a simple proposition, but it seems inevitable that useful information about RPE phenotype development and retention will result. Comparing β-catenin signaling in fetal versus adult human RPE cells might be a useful approach since the former produce more highly pigmented and epithelioid monolayers than the latter.

Cytoskeletal rearrangements and the activity of the Wnt/β-catenin pathway may both be related to the important question of whether RPE phenotype is affected by aging. Ultimately one of our major reasons for interest in RPE morphogenesis is the belief that normal phenotype is essential for optimal cell function, and that loss of RPE phenotype contributes to pathology, especially age-related retinal disease. Here the concept is developed that aging may produce disruptions of RPE phenotype by mechanisms involving oxidative stress (a well established aging influence) as a disrupter of the organization or integrity of the cytoskeleton (a well established determinant of phenotype). Age-related modifications are presumed to result from stress that is sub-lethal and which produces structural modifications that are subtle, affecting what might be called the 'molecular phenotype' rather than the gross morphology of aging cells. Analyzing sub-lethal stress that is revealed as declines in functional efficiency rather than overt cell death is a useful future direction for understanding the effects of age on RPE structure and function. Further, β-catenin participates in a Wnt-related pathway that helps regulate the expression of genes that protect against oxidative stress. Exploration of the Wnt/β-catenin pathway recommended above may therefore aid our understanding not only of re-morphogenesis and re-pigmentation, but also of RPE stress in aging. Activity of the Wnt/β-catenin pathway could provide a unifying theory helping to explain many of the most vexing problems associated with understanding RPE cell structure, function, and contribution to age-related pathology.

Acknowledgements

The author thanks Dr. Brian McKay, University of Arizona, for his helpful suggestions on the manuscript and Dr. Mariusz Zareba for assistance with the studies of organelle motility and with preparation of the figures. Support for research from the author's laboratory was provided by NIH grants R01 EY015284, R01 EY013722 and P30 EY01931, by an unrestricted grant from Research to Prevent Blindness, Inc., and by Milwaukee area donors and foundations supporting macular degeneration research.

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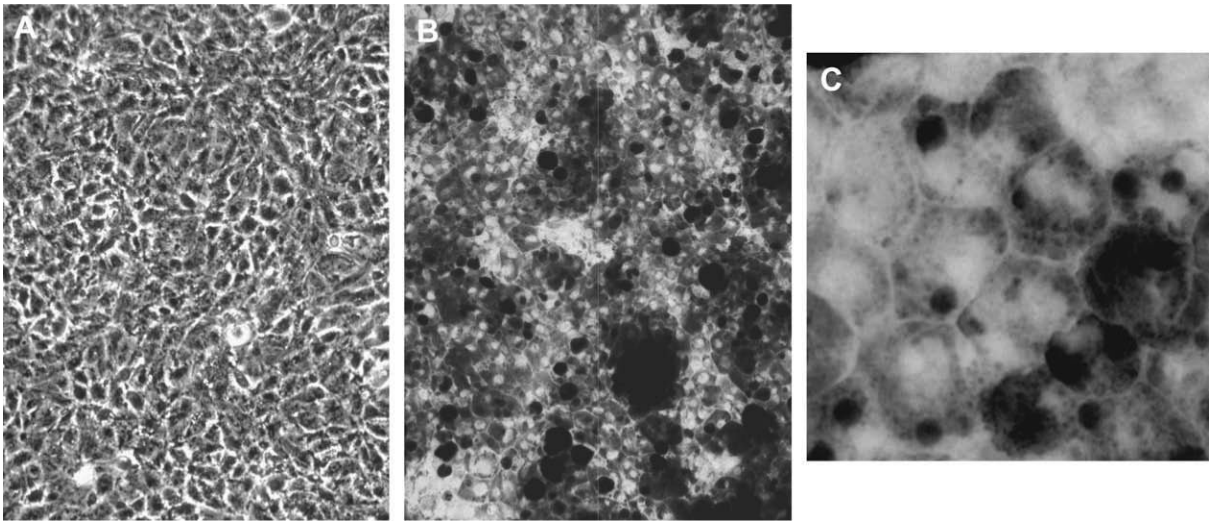

Fig. 1.

Phase contrast micrographs of a human RPE culture from a post-natal human donor that spontaneously melanized after a long post-confluent interval. The culture is shown at two times post-confluency: 1 week (A) and 55 weeks (B). A higher magnification view of the late post-confluency culture is shown (C) to illustrate the epithelioid shape of the cells.

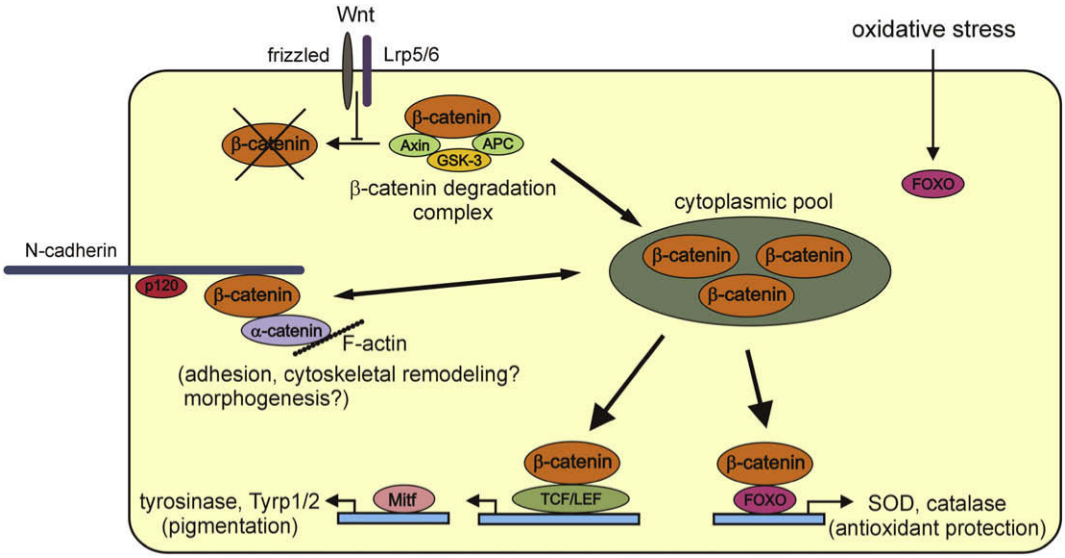

Fig. 2.

Highly simplified diagram of signaling pathways involving β-catenin that may be relevant for RPE cell morphogenesis and susceptibility to age-related oxidative stress. N-cadherin is specified as the cadherin adhesion protein since it is the major cadherin of cultured human RPE cells; its role in cytoskeletal remodeling and morphogenesis is unclear. For more detailed pathway maps, see the Science database of cell signaling, The Signal Transduction Knowledge Environment (STKE), at http://stke.sciencemag.org/cm/. Abbreviations: APC (adenomatous polyposis coli), FOXO (forkhead box O), GSK (glycogen synthase kinase), Lrp (lipoprotein receptor-related proteins), Mitf (microphthalmia-associated transcription factor); TCF/LEF (T cell factor/lymphoid-enhancing factor), Tyrp (tyrosinase-related proteins).

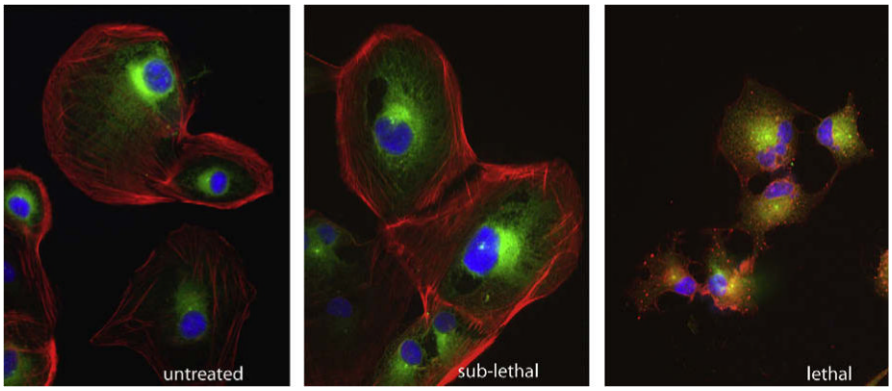

Fig. 3.

Fluorescence micrographs of ARPE-19 cultures showing the effects of blue light-induced stress on the cytoskeleton. Cells are stained with rhodamine-phalloidin to show filamentous actin (red), antibodies to β-tubulin to show microtubules (green) and Hoescht dye for nuclei (blue). The cultures were untreated, or irradiated with violet-blue light (400−410 nm) to produce sub-lethal (4 mW/mm^2) or lethal (10.8 mW/mm^2) injury. Lethality was determined by a dynamic measure of apoptotic blebbing as previously described (Zareba et al., 2007). Lethally damaged cells are less spread with a disrupted cytoskeletal network.

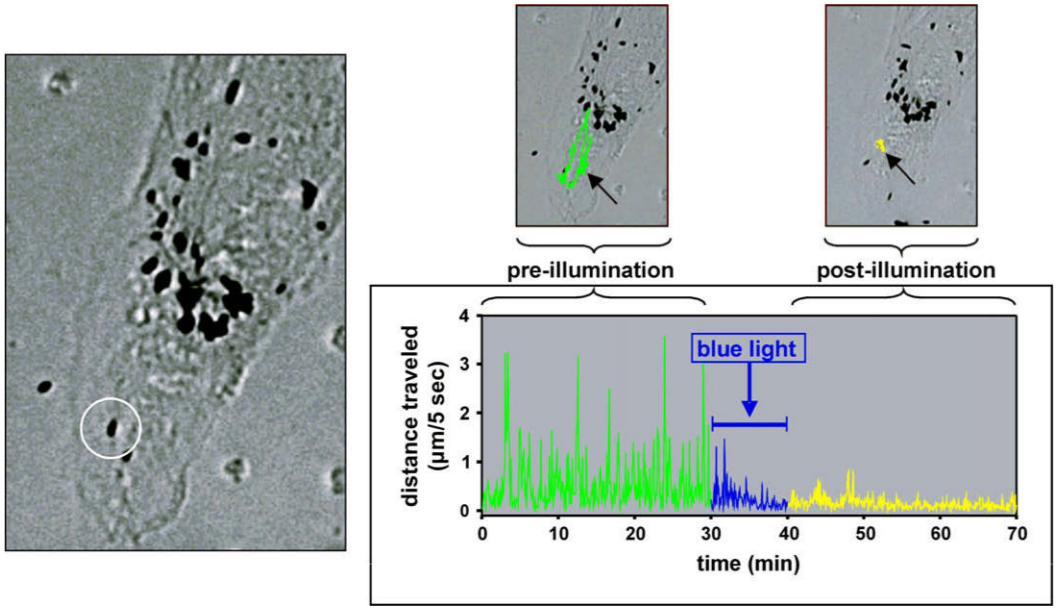

Fig. 4.

Effect of blue light treatment on the motility of phagocytized porcine melanosomes in ARPE-19 cells. Left: phase contrast micrograph showing a granule used for motility tracking (circled). Right: the granule's movement pre-illumination (green) and post-illumination (yellow) shown graphically and by colored traces superimposed on smaller micrographs. For the experiment, cultures were illuminated for 10 min with violet-blue light (400−410 nm at 4 mW/mm^2) delivered via the epi-illumination system of the microscope using a protocol empirically determined to induce sub-lethal photic stress. To track granule movement, images were captured at 5 s intervals; image acquisition and data analysis were performed using Premier MetaMorph software. The blue light effect is quantified by comparing the total distance traveled over 30 min pre-illumination (188 μm) versus post-illumination (45 μm). For the granule illustrated here, motility decreased 76% on blue light treatment.