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Cell Cycle Regulation in the Developing Lens

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Abstract

Regulation of cell proliferation is a critical aspect of the development of multicellular organisms. The ocular lens is an excellent model system in which to unravel the mechanisms controlling cell proliferation during development. In recent years, several cell cycle regulators have been shown to be essential for maintaining normal patterns of lens cell proliferation. Additionally, many growth factor signaling pathways and cell adhesion factors have been shown to have the capacity to regulate lens cell proliferation. Given this complexity, understanding the cross talk between these many signaling pathways and how they are coordinated are important directions for the future.

Keywords

lens; proliferation; cell cycle regulation; growth factors; adhesion factors

1. Introduction

Regulation of the cell division cycle is an essential process by which the cell monitors its growth and differentiation. During the course of embryogenesis, cells exhibit the capacity to quiesce, to proliferate, to irreversibly withdraw from the cell cycle and undergo terminal differentiation. The ocular lens has been an ideal model system for unraveling in a developmental context in vivo the roles of cell cycle regulatory factors in maintaining proper control of proliferation and the signaling pathways in turn regulate the activity of the components of the cell cycle machinery. In recent years, major advances have been made in understanding the mechanisms through which cell proliferation is regulated in the lens. As it is impossible to cover all the work in this area, in this review, I first will focus on our current understanding of the factors required for maintaining cell cycle control in the lens epithelium and as the epithelial cells begin to differentiate into fiber cells. Then, I will focus on the growth factor and cell adhesion signaling pathways that are believed to be involved. Finally, I will discuss factors that may modulate and coordinate the myriad of signaling pathways that have been proposed to impinge on the cell cycle machinery, thereby maintaining the proper patterns of lens cell proliferation and differentiation.

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2. Patterns of cell proliferation in the developing lens

The lens of the postnatal animal consists of two related cell types, the fiber cells, which comprise the bulk of the lens, and the epithelium, which borders the fiber cells on the anterior surface. The formation of the lens during embryonic development in the mouse begins on day E9.5 in embryogenesis, when a group of cells in the head ectoderm thicken, forming the lens placode. Subsequently, these cells invaginate, forming the lens pit. Eventually, by day E10.5-11.0, cells of the lens pit have separated from the overlying head ectoderm, forming the hollow lens vesicle (see Figure 1). Soon thereafter, cells in the posterior in close proximity to the optic cup and under the influence of unique combination of growth factors in the vitreous humor become postmitotic and begin to elongate towards the anterior surface, eventually occluding the lens vesicle. These form the primary fiber cells. The cells directly opposite the optic cup form the epithelium, a monolayer of cells retaining their epithelial structure and proliferative potential. The continued growth of the lens depends on the regulated proliferation of the epithelial cells followed by their migration to the posterior and differentiation into secondary fiber cells (for reviews see [1,2]).

One of the reasons the lens is such an ideal system for studying the mechanisms that underlie cell cycle regulation during development is that patterns of proliferative activity are very well characterized at the histological level. Studies in the 1960's and 1970's [3-9] and early 1980's [10] characterized extensively the regions of high and low proliferative activity as a function of developmental age in numerous models including chick, rat and mouse. These studies showed that DNA synthesis and mitosis occurred throughout the lens at the lens pit and lens vesicle stages. However, soon thereafter, cells in the posterior lose their proliferative capacity as they begin to elongate into primary fibers, thus leaving DNA synthesis and mitosis restricted to the anterior epithelium. As development proceeds through embryogenesis and into postnatal life, a distinct compartmentalization of regions with a high proliferative index and regions with substantially lower proliferative index arises within the epithelium, giving rise to three zones: a central epithelium characterized by low proliferative index with most cells in a quiescent state, a proliferation or germinative zone of high proliferative index just anterior to the lens equator, and a transition zone to the posterior or the equator, where proliferation is not observed. The cell cycle time was estimated to be shortest in the germinative zone and throughout the lens to increase with increasing age. The withdrawal from the cell cycle in cells in the posterior of the lens vesicle and transition zones was correlated with the onset cell elongation and the expression of β -crystallins [8,9]. Although the central epithelium of the postnatal animal exhibits a generally quiescent state, with few cells proliferating at any given time, these cells do retain the proliferative potential. After explanting central epithelial cells from postnatal day 10 rats into tissue culture, a burst of proliferation is observed [11].

The lens grows throughout the life of the animal without appreciable cell loss. Albeit at slow rates in postnatal animals, cells are continuously added to the differentiated fiber cell compartment from the proliferation-competent epithelium. This capacity for self-renewal in the lens, as with other epithelia, must rely on the presence of a stem cell population. According to theory, self-renewing tissues consist of a hierarchy of cells with varying proliferative potential. The stem cell with unlimited proliferative potential divides rarely. When it divides, it gives rise to a transient amplifying cell (TA) that has limited proliferative potential that, upon its final division, enters the postmitotic state (for reviews see [12-14]). A variation on this model, includes two types of TA cells, a young TA cell which possesses high proliferative potential and a mature TA cell, which exhibits low proliferative potential and upon its final division enters the postmitotic state [15].

Where the lens stem cells reside was investigated in 1981 by Rafferty and Rafferty [10] using the mouse as the model organism. Using short term ^3H -thymidine labeling and

autoradiography, they observed that the germinative zone consisted of a population of slowly cycling cells that divided once every 17–20 days giving rise to daughters that moved posteriorly and ultimately into the fiber cell compartment. By contrast, the very small number of radiolabeled central zone cells did not appear to move within the time frame of their analysis. Therefore, it was suggested that the stem cell population resided in the germinative zone and central zone cells divided to facilitate expansion of the epithelium as the lens grows.

Recently, Zhou et al. [16] have revisited the question of where lens stem cells reside using the current experimental techniques of long term labeling followed by long term chasing that are used in the stem cell field. Using this approach, these authors identified the heavily labeled cells only in the central epithelium, suggesting the presence of a stem cell population in this region of the epithelium, and non-label retaining cells, in the germinative zone. Interestingly, they observed lightly labeled cells, presumably the daughters of the heavily labeled cells, in both the central epithelium and germinative zone. The authors suggest that the hierarchy of proliferative cells in the lens includes the stem cell, the young TA cell, the mature TA cell, and the postmitotic cell. They further suggest that the lightly labeled cell may have stem cell character as well as the heavily labeled cells. What role these heavily and lightly labeled cells play in lens biology, whether the lightly labeled cells in the central epithelium migrate into the germinative zone, and which cells in the lens exhibit other characteristics of stem cells are just a few of the unanswered questions.

3. Cell Cycle Regulation in the Lens

3.1 Brief review of cell cycle regulation as learned from in vitro studies

The basis for studies to identify factors that regulate proliferation in the lens comes from extensive in vitro work that identified the major players in cell cycle regulation in somatic cells. This section briefly reviews our current understanding of the factors involved in cell cycle regulation (for a more thorough review, especially as it pertains to the lens, see [17]). The cell cycle is divided into four phases, G1 (first gap), S (DNA synthesis), G2 (second gap) and M (mitosis). Progress through the cell cycle is controlled by checkpoints that ensure the completion of one phase of the cycle before entering the next. Cells can enter a quiescent state if successful completion of a phase has not occurred. Whether to exit G1 phase and enter S phase or withdraw from the cell cycle into a quiescent phase, referred to as G0, is determined at the “restriction point” [18–22].

Progression through the cell cycle is regulated by cyclin/cyclin-dependent kinase (Cdk) complexes. D-type and E-type cyclins function in the G1 phase of the cell cycle [19,22–28]. D-type cyclins associate with Cdk4 and Cdk6 while cyclin E associates with Cdk2 [29]. Cyclin A binds Cdk2 and Cdc2 and is required for both S-phase and the G2/M transition [30–32] while cyclin B/Cdc2 complexes appear to be specific for control of mitotic entry. Cyclin/cdk complexes are in turn negatively regulated by cyclin dependent kinase inhibitors (CKIs). There are two families of CKIs. The p16INK4a family, which includes p15, p16, p18, and p19 [33, 34], bind to and inhibit Cdk4 and Cdk6 kinases. The p21^{CIP1} family of CKIs, which includes p21^{CIP1} (p21), p27^{KIP1} (p27) and p57^{KIP2} (p57), inhibit all Cdks involved in G1/S transition and to a lesser extent cyclin B/cdc2. Many cell cycle regulators are the targets of degradation by the ubiquitin mediated proteolysis system and for example, degradation of cyclin B via this pathway is necessary for exiting mitosis [35].

Cyclin/cdk complexes are responsible for regulating the activity of specific target proteins through phosphorylation. Important targets of the cyclin/cdk complexes are the three members of the retinoblastoma protein family, pRb, p107 and p130 [36,37]. pRb is considered to be the critical G1 phase restriction point factor [38]. The activity of pRb is regulated during G1 phase of the cell cycle by cyclin D/cdk4 or cdk6 and cyclin E/cdk2 complexes. During much of G1,

pRb is in its hypophosphorylated, active form as a consequence of cyclin D/cdk4 or 6 activity [28,39–42] and by the end of G1 phase becomes inactivated by hyperphosphorylation due to the action of cyclin E/cdk2. In its active form, pRb binds to members of the E2F family of transcription factors, primarily E2Fs 1, 2, and 3, and recruits histone deacetylases (HDACs) to the complex [43–45]. This blocks the transcriptional transactivation capacity of the E2F-DP heterodimer [46], thereby repressing transcription of E2F target genes such as *cyclin E*, *dhfr*, *cdc2*, *B-myb*, *c-myc*, and *N-myc* [36,37,46]. When pRb is inactivated, E2Fs are transactivation competent, target gene expression occurs, thereby permitting progression into S phase. The p130 protein is thought to play its role primarily in quiescent cells whereas the p107 protein is thought to play its role primarily in S phase [36,46]. The activities of p107 and p130 are regulated by cyclin/cdk complexes. Whereas pRb appears to preferentially bind to E2Fs 1–3, p107 preferentially binds E2F4 and p130 binds E2Fs 4 and 5 [47–53].

3.2 Expression patterns of cell cycle regulators in the lens

Based on the knowledge discussed in Section 3.1, work on cell cycle regulation in the lens began with the characterization of the expression patterns of cyclins, CDKs, CKIs, pRb proteins and E2Fs. Today, it is appreciated that an extensive array of cell cycle regulators is expressed in the epithelium (see Table 1 for a summary). Of the cyclins, cyclin A [54,55], cyclin B [56], and cyclin D1 and cyclin D2 [54,57,58] are clearly expressed in the epithelium. Low levels of cyclin E also were detected in the lens [59], presumably in the epithelium as proliferating cells are located in this compartment. The Cdk, Cdk2, Cdk4, and Cdc2, were detected in the epithelium [56]. Cdk4 also was detected in the equatorial region where cells stop dividing and start differentiating [54]. Cdk2-associated kinase activity was detected in epithelial cells [60]. Of the CKIs, p16 (H. Pan and A.E. Griep, unpublished data) is expressed throughout the epithelium and p57^{KIP2} is highly expressed in the transition zone [61]. Cdk4-p57 complexes were found in epithelial cells, suggesting a role for this CKI in maintaining normal cell cycling in the epithelium [60]. All three members of the pRb family, pRb, p107 and p130 are expressed [62] (H. Pan and A.E. Griep, unpublished data). In neonatal mouse lens, *Rb* transcripts were found to be most abundant in the equatorial region (H. Pan and A.E. Griep, unpublished data), a pattern reminiscent of the *p57* expression. Transcripts for E2Fs 1–5 are present [62,63]. Thus, a near full complement of the key cell cycle regulators appears to be expressed in the epithelium, in keeping with the capacity of these cells to proliferate. Interestingly, the E2F target gene, *c-myc*, is highly expressed in the epithelium whereas its family member, *N-myc*, is poorly expressed in the epithelium [64].

By contrast, the complement of cell cycle regulators expressed in the fiber cells is more restricted. Of the cyclins, only cyclin B1 [56], cyclin D1 and cyclin D2 [54,57] are expressed in fibers. Cdk4 is present in the equatorial region where cells stop dividing and start differentiating [54]. Cdc2 is found in the fiber cells [56,65]. Interestingly, cyclin B/cdc2 activity was detected in fiber cells [60], suggesting that these factors, which normally are considered to be involved only in cell growth, may also play a role in cell differentiation [65]. Both *p57^{KIP2}* and *p27^{KIP1}* are expressed in fibers [61,66]. Upregulation of *p57^{KIP2}* and *p27^{KIP1}* expression appears to require the activity of the transcription factor Prox1 [67]. In keeping with the postmitotic nature of fiber cells, no cyclin D/cdk4 activity was detected in fiber cells and p57 was found complexed to cdk4 in coimmunoprecipitation assays [60]. The pocket proteins pRb and p107 are expressed in fibers [62]. However, interestingly p130 protein was not found, likely due to its degradation by the ubiquitin-mediated proteasome pathway [62]. Of the E2Fs, E2Fs 1,3 and 5 are expressed [62,63]. The expression of E2Fs normally thought to be only involved in proliferation (i.e. E2Fs 1 and 3) suggests that at least in the lens, they may serve additional roles. Interestingly, while expression of the E2F target gene, *c-myc*, is downregulated with differentiation, the E2F target gene, *N-myc*, appears to be specifically upregulated as cells begin to withdraw from the cell cycle [64].

3.3 Functional analysis of cell cycle regulators in the lens

Based on the known patterns of cell proliferation discussed in Section 2 and on the expression patterns of cell cycle regulators discussed in Section 3.2, the next task was to determine which of these cell cycle regulatory factors are necessary and sufficient for maintaining the normal patterns of cell proliferation in the epithelium and directing cell cycle exit as epithelial cells begin their terminal differentiation into fibers. Analysis of transgenic and mutant mice has been critical in addressing this goal.

The knowledge that pRb is an important regulator of the cell cycle *in vitro* and that it is expressed in the lens prompted investigators to ask if pRb is required for cell cycle withdrawal during fiber cell differentiation. One approach taken was to generate transgenic mice that expressed dominant repressors of pRb function under the control of the α A crystallin promoter. The E7 oncoprotein from Human papillomavirus type 16 has the capacity to bind to and inactivate the hypophosphorylated form of pRb [68,69]. A C-terminally truncated SV40 large T antigen similarly binds to pRb but fails to bind to p53 [70]. Transgenic mice expressing the E7 oncoprotein [71] or a C-terminally truncated large T antigen from SV40 [72] under the direction of the murine α A crystallin promoter were generated. Lens cells in the transition zone in these transgenic mice failed to withdraw from the cell cycle and elongate. Instead, these abnormal cells showed a high propensity for apoptosis [71,72]. The *Rb*-null mice generated at this same time exhibited a similar lens phenotype [73]. The failure to exit the cell cycle in the *Rb*-deficient cells was correlated with upregulation in expression of *E2Fs 1-3* [55,63] and *E2F* targets such *cyclin E* [54], *cyclin A* and *p19Arf* and a downstream factor, *p53* [55]. Upregulation of *p53* targets such as *p21* and the pro-apoptotic gene *Bax* was also observed (M.M. Nguyen, R.K. Hyde and A.E. Griep, unpublished data). Transgenic overexpression of *E2F-1*, *E2F-2*, *E2F3a*, and *E2F4* recapitulated, at least in part, the lens phenotype in the *Rb* mutant mice [74,75]. By crossing *Rb*-null or E7 transgenic mice to *E2F-1*, -2, or -3 null mice, it was demonstrated that the continued cell proliferation depended at least in part on these *E2F* factors [76,77].

Apoptosis can occur through both *p53*-dependent and *p53*-independent pathways. Many genes falling into the *p53*-dependent apoptosis pathway such as *E2F-1*, *p19Arf*, *p53*, and *Bax* were found to be upregulated in the lenses of the E7 transgenic mice [55], suggesting that apoptosis was *p53*-dependent. By crossing *Rb*-null or E7 mice to *p53*-null mice, it was learned that early in lens development, at day E13.5, apoptosis was almost entirely *p53*-dependent [73]. However, by day E17.5, both *p53*-dependent and *p53*-independent pathways were activated [78]. At present time the nature of the *p53*-independent pathway has not been elucidated. It is possible that *p53* family members, *p63* and *p73* which have been documented to play a role in apoptosis [79,80] may be involved. Also, it has been observed to co-expression of the viral oncoprotein E6 with E7 rescues apoptosis to a much greater extent than the *p53*-null mutation [78]. Further mutational analysis of the role of cellular proteins targeted by E6 may provide some clues as to components of the *p53*-independent pathway. Finally, it would be predicted that *p19Arf* and *Bax* would be part of the *p53*-dependent apoptosis pathway. Although loss of *Bax* partially rescued the apoptotic defect in the lenses of the E7 transgenic mice (M.M. Nguyen and A.E. Griep, unpublished data), loss of *p19Arf* provided very little rescue of apoptosis in the lenses of the *Rb*-null mice [81]. Overall, work to date shows that while some parts of the model derived from tissue culture studies hold in the lens, clearly additional factors and pathways must be involved. One clear hole in our knowledge is whether the pRb family members play any role in maintaining the postmitotic state of fiber cells. Since p107 can be detected in complexes with E2F in fiber cells [62], p107 is an obvious candidate. However, p107 alone is not essential when pRb is intact, as *p107*-null mice exhibit normal lenses. It has been proposed that p107 can in part compensate for loss of pRb [82] and recent analysis

suggests this may be the case in the lens fibers (R.K. Hyde, S. Hanson and A.E. Griep, unpublished data).

Knowing that pRb is essential for cell cycle withdrawal, further studies sought to elucidate the required upstream regulators that would keep pRb in its active, hypophosphorylated form. The CKI, p57^{KIP2}, has been suggested to be the main effector that downregulates cyclin/Cdk activity to activate pRb, based on its high levels of expression in cells that are beginning to differentiate in the equatorial region of the lens [61,66,83]. Loss of p57^{KIP2} in mice caused a slight increase in the proliferation rates of these cells, much less severe than what has been observed in mice lacking *Rb*, suggesting that, while this CKI may contribute, other mechanisms may be involved or other factors compensate for p57^{KIP2} loss [84]. Loss of p27^{KIP1} alone did not cause lens abnormalities. However, the combined loss of p27^{KIP1} and p57^{KIP2} resulted in hyperproliferation and apoptosis of lens fiber cells [61] bearing strong similarities to the effect on these cells in the *Rb*-null lenses, suggesting these two CKIs cooperatively activate pRb during the differentiation of lens epithelial cells into fiber cells. Recent analysis of lenses from mice carrying mutations in both *Rb* and p57^{KIP2} suggest that at least at day E13.5 in embryonic development, p57 is an upstream regulator of pRb. (R.K. Hyde, S. Hanson and A.E. Griep, unpublished data). Moving further upstream, in mice lacking *Prox1*, upregulation of p57^{KIP2} and p27^{KIP1} expression did not occur and phenotypically and lenses resembled the lenses of the *Rb* null mice [67], suggesting that *Prox1* is upstream of p57^{KIP2} and p27^{KIP1}. Thus, genetic analysis has begun to map the pathway leading to cell cycle exit as lens epithelial cells differentiate into fibers. Further studies will be needed to understand if the requirement for *Prox1* in p57^{KIP2} and p27^{KIP1} expression is due to its direct transcriptional regulation of the promoters of these CKI genes.

In contrast to our extensive understanding of the mechanisms of cell cycle regulation in lens cells as they leave the cell cycle to differentiate into fibers, our understanding of the mechanisms that control the cell cycle in the epithelium is much more limited. As reviewed in Section 3.2, expression of the three *Rb* family members, *E2Fs 1-5*, and at least 2 CKIs, *p16* and p57^{KIP2}, have been detected in the epithelium. Given that the epithelium consists of regionally specified groups of cells that exhibit different proliferative behaviors, it would be reasonable to assume that multiple pocket proteins, E2Fs and CKIs would be required for cell cycle control, as defined by genetic analysis, and that there would be overlap in function or compensation in the absence of one factor. This prediction is consistent with the results from the analysis of the mice nullizygous for any one *Rb* family member [73,85,86], *E2F* [77] or *CKI* [61], or mice designed to overcome the early embryonic lethality of the *Rb*-null mutation such as *Rb*-null rescue chimeras [87–89], or mice carrying *Rb* minigenes [90]. Loss of all three *Rb* family members recently has been shown to be necessary for abrogating the G1 checkpoint in cultured mouse embryonic fibroblasts [91,92] and pRb and p107/p130 appear to be required for regulation of different sets of genes [93]. Thus, if pocket proteins are essential for maintaining cell cycle control in the epithelium, then it may be necessary to interfere with the function of all three simultaneously. Consistent with this hypothesis, transgenic expression of the HPV-16 oncoprotein E7, which can bind to and inactivate all three pRb proteins, in the epithelium of postnatal mice resulted in an increased rate of proliferation throughout the epithelium and this capacity of E7 was dependent on intact pocket protein binding and degradation motifs [94]. Interestingly, it appears that the pocket protein requirements may be temporally regulated. Recent studies showed that when *Rb* was conditionally inactivated in the lens at the lens vesicle stage by cre/lox techniques, proliferation rates in the lens epithelium at day E13.5 were abnormally low and this defect could be rescued by simultaneous loss of *p130* or *p107* (R.K. Hyde, S. Hanson and A.E. Griep, unpublished data). Thus, in contrast to the enhancement of the cell cycle defect in fiber cells that is observed with simultaneous loss of *Rb* and *p107*, loss of a second pocket protein in the epithelium suppresses the effect of loss of *Rb*, returning proliferation rates to normal. Interestingly, the mechanism may involve the

action of p57, as loss of *p57* partially rescues the defect in cell cycle regulation resulting from loss of *Rb* (R.K. Hyde, S. Hanson and A.E. Griep, unpublished data). Further studies will be required to fully understand the mechanisms involved.

4. Growth factor signaling pathways regulating lens cell proliferation

4.1 Growth factors and their receptors affecting lens cell proliferation

From the time of the initial differentiation of cells in the posterior of the lens vesicle throughout the remainder of the life of the animal, the lens maintains its polarized structure, with the epithelium situated at the anterior, facing the aqueous humor and cornea, and the differentiating fibers situated at the posterior, facing the vitreous humor and retina. The polarized nature of the lens is thought to be regulated by the ocular environment, as was suggested by the lens inversion experiments of Coulombre and Coulombre [95]. Hence, growth factors in the environment are thought to be responsible for maintaining epithelium and fibers. Growth factors including FGF-1 and FGF-2 [96], PDGF [97], PDGF-D [98], insulin/IGF1 [99–101], EGF/TGF α [101,102], and HGF [103,104] all have been shown to stimulate lens epithelial cell proliferation in the cultured epithelial cells of a variety of species including chick, rat and human, epithelial explant cultures, or whole lens cultures (for review see [105]). On the other hand, TGF β has been shown to inhibit the proliferation of human lens epithelial cells in culture [106]. Epithelial cells in rat explants have a particularly interesting response to FGFs in that a low concentration of FGF stimulates cell proliferation while a higher concentration of FGF stimulates fiber cell differentiation [96]. Based on these studies and studies documenting the higher levels of FGF in vitreous as opposed to aqueous [107] arose a model in which a posterior to anterior gradient of FGF stimulation exists within the eye, thereby instructing cell cycle exit and differentiation in the posterior as opposed to proliferation in the anterior.

Many of these growth factors and their cognate tyrosine kinase receptors have been demonstrated to be expressed in the eye and/or lens in a pattern that would support the contention that the particular growth factor pathway could be involved in regulating lens cell proliferation (for review see [105]). For example, *PDGF-A* and its receptor *PDGFR- α* are expressed in the proliferating cells of the epithelium [108]. *PDGF-D* is expressed in iris and ciliary body and this growth factor is found in aqueous humor [98]. *FGFs -1* and *-2* are expressed in the lens as well as in surrounding ocular tissues [109]. *FGFR-2* is highly expressed in the epithelium whereas *FGFRs -1* and *-3* appear to be more highly expressed in the transition zone [110]. Receptors for TGF β are expressed in the lens [111,112].

Transgenic and gene disruption studies have provided evidence for the capacity for several growth factor signaling pathways to affect lens cell proliferation and differentiation. However, in some instances possible conflicting results have arisen as well. Overexpression of *PDGF-A* [108] led to abnormally high levels of DNA synthesis across the epithelium and increased G1 phase (cyclin D2) and S/G2 phase (cyclin A) markers across the epithelium [108]. Although overexpression of *PDGF-A* was sufficient to induce hyperproliferation [108], the lenses of *PDGFR- α* null mice did not show any obvious abnormalities [113]. Overexpression of *IGF-1* led to hyperproliferation and an expansion of the germinative and transition zones of the epithelium towards the posterior [114]. That overexpression of *IGF-1* promotes proliferation in the mouse lens is distinctly different from its effect in chick culture systems where it has been shown to induce fiber cell differentiation [99]. Analysis of the role of TGF β 1 in lens cell proliferation in transgenic models has shown that overexpression of this growth factor, rather than suppressing normal epithelial cell proliferation during development, was associated with supporting the growth of epithelial cells that had undergone epithelial to mesenchymal transition in postnatal mice [115]. Transgenic expression of dominant negative TGF β receptors showed that although cataracts formed, proliferation was unaffected [116]. As redundancy in the pathway could affect the ability to detect a specific effect of TGF β on lens

cell proliferation, further analysis is needed to clarify the role of this growth factor pathway in regulating normal lens cell proliferation. More promising appears to be the possibility that one of the BMP receptors, *Alk3*, may mediate a BMP-dependent effect on epithelial cell proliferation, as conditional deletion of this receptor at the lens placode stage resulted in a decreased rate of DNA synthesis [117].

Overexpression of several different *FGF* ligands stimulated cell cycle exit and differentiation in the anterior epithelium [118,119] suggesting these factors are sufficient to force cell cycle exit and overexpression of a secreted *FGFR3*, which is predicted to act as an inhibitor of FGF signaling, inhibited differentiation and supported proliferation [120]. However, analysis of mice with null mutations in FGF ligands has failed to identify a particular FGF responsible for modulating lens cell proliferation, suggesting that no one factor is sufficient or that the specific FGF involved has not been identified. Loss of *FGFR-1* [121] or *FGFR-3* [122] in the lens did not affect lens epithelial cell proliferation or exit from the cell cycle. However, targeted disruption of *FGFR2* at the lens placode stage using cre/lox technology showed that, while this receptor is not required for epithelial cell proliferation, it is required for lens epithelial cells to exit the cell cycle during differentiation, for correct patterns of expression of the p27 and p57 and for lens cell survival [110]. Since, growth factor families such as FGF consist of multiple ligands and receptors, it is perhaps not surprising functional redundancy or compensation may preclude a strong defect arising as the consequence of loss of function of a single ligand or receptor. Additionally, compensation could extend beyond a given growth factor family given that so many different growth factors have the capacity to influence lens cell proliferation. Future studies will need to be undertaken to gain insight into the cross-talk within and between these growth factor families.

4.2 Downstream signaling effectors of growth factor stimulation of lens cell proliferation

Growth factors affect cell proliferation through the activation of a number of downstream signaling cascades, which ultimately impact on gene expression. Engagement of growth factor receptor tyrosine kinases by ligand frequently leads to the activation of the downstream signaling pathways, Raf/MEK/ERK and/or PI3K/AKT. Numerous studies have addressed the involvement of these pathways as downstream mediators of specific growth factors in the regulation of lens cell proliferation. In rat explants, aqueous humor, FGF, PDGF, IGF and EGF stimulation of cell proliferation has been shown to be dependent on both ERK and PI3K activation [123] as is FGF-induced differentiation, which may reflect an FGF-dependent cell cycle exit through upregulation of p57. Interestingly, in chick culture systems, FGF induced epithelial cell proliferation has been shown to be independent of ERK activation whereas IGF induced proliferation is dependent on ERK activation [124,125], suggesting the possibility of species specificity in the response or differences due to the experimental conditions. Other signal transduction cascades have been implicated in lens proliferation. Many members of the JAK/STAT pathway are expressed in lens epithelial cells [126,127]. STAT3, and to a lesser extent STAT1, has been shown to be activated in response to treatment of mouse lens epithelial cells with growth factors such as PDGF and IGF-1 [127] and to treatment of chick lens epithelial cells with PDGF and GM-CSF [126]. However, lens specific deletion of STAT1 or STAT3 using cre/lox technology showed that neither was essential for normal lens development, suggesting the possibility of redundancy between family members. Finally, HGF has been shown to promote proliferation of human immortalized lens epithelial cells via ERK and JNK pathways [103].

Arachidonic acid and/or certain of its metabolites and physiological levels of reactive oxygen species (ROS) have been implicated as intermediates in certain growth factor signaling pathways. For example, arachidonic acid has been shown to be an intermediate in the growth stimulatory response of human lens epithelial cells, HLE-B3, to PDGF. It was shown that in

this pathway arachidonic acid stimulates a transient increase in the concentration of intracellular ROS which then led to increased levels of activated ERK and JNK [128,129]. The arachidonic acid metabolite 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE) was shown to work in synergy with EGF to activate PKC β and promote proliferation of primary human lens epithelial cells [130,131]. Proliferation of N/N1003A rabbit lens epithelial cells requires 12-lipoxygenase dependent metabolite(s) of arachidonic acid for proliferation [132]. Another important intermediate in the EGF signaling pathways is calcium, which is released from its endoplasmic reticulum stores in response to EGF treatment (for a review on calcium signaling in the lens see [133]). Depletion of the calcium stores in FHL124 human lens epithelial cells through treatment with thapsigargin blocked the EGF-dependent activation of ERK and cell proliferation [102]. Stimulation of FHL124 cells with EGF resulted in increased levels of total and activated CDK2 and reduced levels of p27^{KIP1} while depletion of the calcium stores either with or without treatment of the cells with EGF had the opposite effect [102].

Transgenic mouse experiments have been carried out to address the role of Ras in lens development. Overexpression of activated *H-ras* in the lens epithelium and fibers using a δ -crystallin/ α A crystallin fusion promoter led to increased rates of cell proliferation in the epithelium; however differentiation was not affected [134] and conversely, expression of a dominant negative Ras led to hypoplasia in the epithelium that was correlated with reduced levels of activated ERK [135]. It is not clear why manipulation of Ras activity did not affect differentiation given that both proliferation and differentiation are associated with increases in levels of activated ERK. Since the lens expresses three forms of Ras, *H-ras*, *K-ras*, and *N-ras* [134], one explanation could be that the specific form of Ras that was used for the transgene, *H-ras*, has different activities than the more abundant *N-ras*. Alternatively, it is possible that expression levels of the transgene were insufficient to induce the higher levels of activated ERK necessary for differentiation. A third explanation is that the differentiation is activated through a Ras-independent pathway. As the interplay between the many growth factors and downstream signaling pathway that can affect lens cell proliferation is expected to be complicated, it will be necessary to address how these different pathways are coordinated to regulate lens cell proliferation and differentiation.

5. Cell adhesion molecules regulating lens cell proliferation

Cell-cell interactions are thought to elicit signaling pathways that are critical to the regulation of cell proliferation and differentiation. In the lens, evidence suggests that cadherin-mediated signaling events contribute to regulating these cell behaviors. Multiple cadherins are expressed in the chick and mouse lens and each exhibits distinctive patterns [136,137]. Studies in the chick utilizing N-cadherin function blocking antibodies have shown that formation of stable N-cadherin junctions through increased association with the actin cytoskeleton is necessary for the proliferation to differentiation switch [138]. One signaling factor family that is involved in the formation of stable N-cadherin junctions is the Src kinases. Expression of constitutively active Src inhibits junction assembly [139] and blocks the ability of lens epithelial cells to exit the cell cycle [140] while inhibition of endogenous Src kinase activity promotes junction assembly and results in upregulation in expression of the CKIs, p27 and p57 [141]. Together, these studies lead to the hypothesis that down-regulation of Src activity leads to N-cadherin junction formation, which then leads to upregulation of p27 and p57 and exit from the cell cycle. Conversely, therefore, Src activity, through its down-modulation of N-cadherin junction assembly, is required for maintaining proliferation in lens epithelial cells.

Cell-matrix interactions, through the interaction of integrins with their extracellular matrix (ECM) ligands, are also thought to promote adhesion, to impact on cytoskeletal organization and to elicit signaling events that impact on the regulation of growth and differentiation [142]. For example, integrin signaling via FAK and Src is linked to activation of JNK and

progression through the cell cycle perhaps due to upregulation of *c-jun* expression [143,144]. From studies in chick and mouse, it is understood that the various integrin α and β subunits have specific patterns of expression within the lens [142,145–147]. The $\beta 1$ subunit is prominent in both epithelial and fiber cells [145] suggesting that this subunit which can bind to multiple α subunits may be involved in several different functions in the lens. Interestingly, the $\beta 1$ subunit is located not only basal surfaces of the cells that are in contact with the ECM but also on the lateral and apical surfaces, suggesting a role in cell-cell interactions [148]. Of the α -integrin subunits, $\alpha 5$ integrin is highest in the epithelium, as is fibronectin. Therefore, it is thought that the $\alpha 5\beta 1$ integrin, as the fibronectin receptor, may play a role in cell proliferation [142,148]. However, a requirement for this integrin receptor in regulating epithelial cell proliferation has not been addressed to date, nor has the activation of the downstream signaling pathways by this integrin such as the ERK and JNK pathways been fully evaluated in the lens. One possible target is *c-jun* since it has been shown that overexpression of this JNK target stimulates lens epithelial cell proliferation [149].

On the other hand studies in chick and mouse suggest a connection between $\alpha 6$ integrin, its downstream signaling pathway, and cell cycle withdrawal. It has been shown that $\alpha 6$ integrin becomes more highly associated with the cytoskeleton in the equatorial region where cells exit the cell cycle [146]. Furthermore, the isoform of $\alpha 6$ integrin that predominates switches from the $\alpha 6A$ isoform to the $\alpha 6B$ isoform, similar to other differentiation systems [146,147]. Finally, in chick, $\alpha 6$ integrin was found to be associated with signaling intermediates [150], the IGFR-1 receptor and activated ERK [151]. Collectively these data show a possible connection between $\alpha 6A$ signaling, cytoskeletal linkages and cell cycle exit. In the future, studies will need to be undertaken to address the requirements for this integrin in cell cycle regulation.

6. PDZ proteins: coordinators of pathways regulating lens cell proliferation?

Over the past several years, much has been learned about the cell cycle control in the lens. What has emerged is that a multitude of signaling pathways can affect the cell cycle progression or withdrawal in this tissue. How cross talk between these pathways is achieved so that they coordinately regulate lens cell growth and differentiation is largely unknown. One example, that of the $\alpha 6$ integrin complexing with IGFR1, provides evidence that cross talk does exist. One group of proteins that may participate in coordinating various signaling pathways is the PDZ (PSD95/Dlg/ZO-1) domain containing proteins. These proteins, though their protein-protein recognition modules, the PDZ domain, are thought to act as scaffolding proteins, thereby facilitating the formation of supramolecular signaling complexes. The PDZ proteins Dlg and Scrib, in *Drosophila* are required for maintaining cell adhesion, polarity and proliferation in embryonic epithelia such as the imaginal discs. Mutations in either gene result in a loss of these characteristics and neoplastic over growth of the disc [152,153]. Recent studies suggest that the Dlg-1 and Scrib, the mouse homologs of the *Drosophila* proteins, may play a role in regulating cell adhesion, polarity, and proliferation and differentiation in the lens. It has been observed that Dlg-1 and Scrib show distinct patterns of overlap with N-cadherin, β -catenin and α -catenin [154]. Furthermore, transgenic expression of the HPV-16 E6 protein in the lens epithelium of postnatal mice (*K14E6* mice) led to hyperplasia, a multilayered epithelium and abnormally differentiating cells that retained proliferative capacity and failed to properly elongate [155]. The HPV-16 E6 protein has the capacity to bind to and interfere with the function of many cellular factors including several PDZ proteins [156–162]. There was no lens phenotype in transgenic mice expressing a PDZ-ligand deficient mutant of E6 in the epithelium (*K14E6 ΔC* mice), suggesting that interference with PDZ protein function was the genesis of the phenotype. The results of genetic interaction studies suggest that the PDZ proteins targeted by E6 and the pRb protein interact in a common or intersecting pathway that affects cell cycle withdrawal (Figure 2). Dlg-1 appears to be at least one of the E6 PDZ targets that may be involved in regulating lens cell proliferation. The lenses in mice carrying a gene trap insertion

in *Dlg-1* [163] showed hyperproliferation throughout the epithelium as well as a failure of cell cycle withdrawal of cells in the transition zone [155]. Also, the lenses of mice carrying a hypomorphic allele of *Dlg-1* exhibited hyperproliferation (C. Rivera and A.E. Griep, unpublished data). Together, these recent studies highlight a new class of proteins that affect proliferation and differentiation in the lens. Future studies will be required to elucidate the individual roles of the various PDZ proteins in lens development and the mechanisms involved that link these proteins to the cell cycle machinery. The presence of PDZ ligands in $\alpha 5$ and $\alpha 6$ integrin and in β -catenin and the knowledge that in *Caenorhabditis elegans* PDZ proteins have been shown to associate with the homolog of the EGF receptor [164,165] suggest various mechanisms through which PDZ proteins could regulate cell growth and differentiation. As the proper regulation of lens cell proliferation and differentiation likely depends which signaling factors are assembled on the scaffold, it will be important to learn how PDZ proteins might coordinate the spatial and temporal assembly of specific macromolecular complexes that would be required.

7. Concluding Remarks

Remarkable progress has been made in recent years into elucidating the factors responsible for maintaining cell cycle control in the developing lens and the signaling pathways that provide the environmental cues necessary to maintain the proper proliferative characteristics in this polarized tissue. These advances have arisen through the use of the powerful combination of in vitro studies, where one can manipulate specifically one factor at time in isolation from the environment, and in vivo approaches, where one can genetically manipulate a given factor in the correct environmental context. Studies have identified many factors that have the potential to be key regulators of lens cell proliferation. Particularly significant is the emergence of a pathway that is required for regulating cell cycle withdrawal that includes pRb, the CKIs, p57 and p27, the transcription factor Prox1, and FGF signaling. Our understanding the complexity of cell cycle regulation in the proliferation competent epithelium, while much more rudimentary, is beginning to increase. Also significant is the demonstration that cadherin and integrin complexes and their associated signaling pathways affect lens cell growth and differentiation and the possibility PDZ proteins such as the mouse homologs of *Drosophila* tumor suppressors Dlg and Scrib also may play a role. Despite the progress, much more remains to be learned about the pathways regulating lens cell growth and differentiation. With multiple growth factor signaling pathways and their intermediates capable of influencing lens cell proliferation, it will be important to determine how all these signaling pathways are coordinated. Genetic approaches to pathway analysis has the potential to provide insight into the relationship between the myriad of signaling pathways that impact on the cell cycle regulation in the lens.

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Figure 1.

Schematic depiction of the patterns of cell proliferation in the mouse lens at different stages of lens development. (A). In the lens vesicle stage at embryonic day E11.0- E11.5, nearly all cells in the lens vesicle have the capacity to proliferate (indicated by the red nuclei) but some in the extreme posterior may have already begun to withdraw from the cell cycle (black nuclei). (B). By approximately day E13.0, primary fibers have occluded the lens vesicle. Proliferation is restricted to the anterior epithelium (red nuclei), as the primary fiber cells and cells in the transition zone have withdrawn from the cell cycle permanently (black nuclei). (C). After birth, the region of high proliferative activity is restricted to the germinative zone (cells with red nuclei) whereas newly differentiating secondary fibers in the transition zone are postmitotic (cells with black nuclei). In the central epithelium, most cells are in a quiescent state (cells with blue nuclei), although an occasional proliferating cell is observed. C, cornea; CB, ciliary body; CE, central epithelium; R, retina; TZ, transition zone.

Figure 2.

Enhancement of the proliferation defect in lenses of *K14E6* transgenic mice by Rb deficiency. *K14E6* transgenic mice were crossed to *Rb*^{+/-} mice. Progeny at postnatal day 6 mice were injected with BrdU one hour prior to sacrifice and BrdU incorporation into newly synthesized DNA was detected immunohistochemical analysis. Shown are representative micrographs of lenses from *Rb*^{+/-}, *K14E6*; *Rb*^{+/+}, *K14E6*; *Rb*^{+/-} mice from this BrdU analysis. The %BrdU incorporation for *Rb*^{+/-} mice was indistinguishable from *Rb*^{+/+} (not shown). Note the increased numbers of BrdU⁺ nuclei in the lenses (most notably in the fiber cell compartment) of *Rb*^{+/-}; *K14E6* mice as compared to *Rb*^{+/+}; *K14E6* mice. Analysis of lenses from *K14E6*^{ΔC} mice in which the PDZ ligand of E6 was deleted, and *Rb*^{+/-}; *K14E6*^{ΔC} mice showed that the %BrdU⁺ cells in lenses of these two genotypes of mice were indistinguishable from *Rb*^{+/+} mice (not shown), indicating that increased proliferation in the lenses of the *K14E6* mice was dependent on the ability of E6 to interact with PDZ proteins.

Table 1
Expression Patterns of Cell Cycle Regulators in the Lens

	Epithelium	Fibers	References
Cyclins	A, B, D1, D2, E ^a	B, D1, D2	54–59
Cdks	Cdk2, 4, Cdc2	Cdk4, Cdc2	54,56,60
CKIs	p57 ^b , p16	p57, p27	60,61,66, Pan and Griep, unpublished
pRb proteins	pRb ^c , p107, p130	pRb, p107	62, Pan and Griep, unpublished
E2Fs	1, 2, 3, 4, 5	1, 3, 5	62,63

^aPresumed to be epithelial specific due to very low expression level and function in cycling cells

^bPresent in anterior epithelium at much lower level than in equatorial and transition zones

^cPresent in anterior epithelium at much lower level than in equatorial and transition zones in lenses of neonatal mice