Corneal development associated with eyelid opening

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ABSTRACT The development of the cornea as a tissue initiates as early as five weeks in the human embryo. This development continues gradually until the time of eyelid opening, which is associated with major developmental changes. These events, most easily observed in rodents, which are born with closed eyelids, include alterations in the rate of cell proliferation in the epithelium, stroma and endothelium; differentiation of the epithelium; appearance of a tear film and tear-film-associated proteins; and swelling and thinning of the stroma. Eyelid opening is also associated with numerous alterations in gene expression. These events are the subject of this review. Readers are directed to the article by Wolosin *et al.*, also in this volume, for an in-depth discussion of early corneal development.

KEY WORDS: *cornea, eyelid, corneal development, corneal epithelium, stroma*

Corneal structure and function

The adult cornea (Fig. 1) consists of a stratified non-keratinizing epithelial cell layer, a thick highly aligned collagenous stroma interspersed with keratocytes, and a single cell layered endothelium. The epithelium sits atop a basement membrane and the endothelial layer resides on a thickened basement membrane, termed Decemet's membrane. In addition, primates and some birds exhibit an acellular layer (approximately 10μm thick) beneath the epithelial basement membrane that is termed Bowman's layer or membrane. This term is frequently misused to refer to the epithelial basement membrane. Through the combination of these various cell layers, the cornea performs three main functions: protection, transmittance of light, and refraction of light. Indeed, the cornea is the main lens of the eye providing 70-75% of the refractive power (Hogan *et al.*, 1971; Jakobiec and Ozanics, 1982; Maurice, 1985).

Corneal organogenesis

Corneal organogenesis has been extensively reviewed in chicks by Hay (Hay, 1979) and in humans by Sevel and Isaacs (Sevel and Isaacs, 1988), and Barishak (Barishak, 2001). In brief, the vertebrate cornea begins its development as the ectoderm overlaying the lens. This primitive epithelium, two cells in thickness, is first apparent at about five weeks in the human embryo and is contiguous with the surface ectoderm. During the next one-to-two weeks in humans, the epithelium stratifies to three-to-four cell-layers thick, the lens completes its formation and detaches from the ectoderm, and the eyelids form and fuse. Almost immediately after the separation of the lens from the corneal epithelium, waves of neural crest cells migrate into the space between the lens and epithelium. These cells become the corneal endothelium and the stromal keratocytes. This migration of cells appears to be species specific. For example, in reptiles, birds, and primates, including humans, two waves are observed - first, endothelial cells then keratocytes. However, in rodents, cats, rabbits, and cattle, a single migration of cells, resulting in both cell types, is observed (Cintron *et al.*, 1983). In the chick, the epithelial cells secrete a collagenous matrix (termed the primary stroma) prior to the influx of neural crest cells (Hay, 1979). This primary stroma is not observed in rabbits, mice, or primates (Cintron *et al.*, 1983).

Following fusion of the eyelids, the primitive corneal epithelium decreases to two cell layers and remains constant until the time of eyelid opening (twenty-four weeks in humans). During the period of eyelid closure, the cornea gradually enlarges and matures. Upon eyelid opening, a number of developmental events occur that result in a mature cornea. These events form the basis of this review.

Morphological changes associated with eyelid opening

Eyelid opening appears to stimulate similar events in all species that undergo the process of eyelid fusion and subsequent opening.

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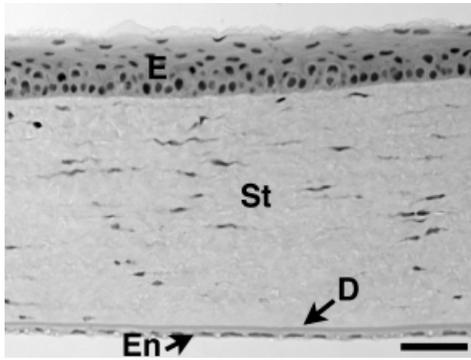
Fig. 1. Cross-section of the mature rat cornea. *The epithelium (E) forms the anterior-most portion of the cornea and serves to protect the remainder of the cornea from fluid loss and pathogen penetration. The epithelium is adherent to a basement membrane. Subjacent to the epithelium is a thick stroma (St) consisting of numerous collagen lamellae organized in an orthogonal pattern. Interspersed between the lamellae are stromal fibroblasts, termed keratocytes. The organized pattern of the stroma provides strength and allows light to be transmitted with minimal distortion. Descemet's membrane (D) is a thickened basement membrane secreted by the endothelium (En). The endothelium is a single-cell layer that functions to pump water out of the stroma. Bar, 50 µm.*

However, it is most easily studied in animals such as rodents, where eyelid opening occurs after birth.

Corneal epithelium

In the adult, the main function of the corneal epithelium is to serve as a protective barrier against fluid loss and pathogen penetration. As seen in Fig. 2, the corneal epithelium in rodents undergoes a remarkable level of differentiation and development that initiates just prior to eyelid opening (twelve-fourteen days-of-age in the rat) (Chung *et al.*, 1992; Watanabe *et al.*, 1993). Until seven days-of-age, only one or two layers of cells are present in the central cornea, the limbus, and the conjunctiva (Chung *et al.*, 1992). At ten days-of-age, epithelial thickness increases to two-to-three layers in all areas. At two weeks-of-age, immediately following eyelid opening, four-to-five cell layers are present in central cornea. The thickness of the epithelium is nearly double that seen at ten days. The thickness continues to increase to five-to-six layers at three weeks, and by four weeks, the thickness reaches its adult level of six-to-seven cell layers. This increase in epithelial thickness has also been observed in developing mouse (Song *et al.*, 2003) and human (Sevel and Isaacs, 1988) corneas. In mouse, the epithelium increases in thickness from 12.7 µm at day one to 49.3 µm at day thirty (Song *et al.*, 2003). Similar changes have been observed in the limbal and conjunctival epithelia (Chung *et al.*, 1992). Along with the change in the number of cell layers, a fundamental change in the shape of the basal cells has also been observed. Until ten days, the basal cells exhibited a flattened, ovoid appearance; however, immediately following eyelid opening, the basal cells became more rounded or cuboidal. At three weeks, the basal cells changed in shape from cuboidal to columnar (Chung *et al.*, 1992). Interestingly, these changes only occurred in the central cornea, not in the limbal or conjunctival epithelium. Fundamental changes in the stratified layers of the epithelium also occur during this period. As cells leave the basal cell layer, they begin to flatten and appear to form more intracellular junctions, termed desmo-

somes. Three-to-four layers of these cells (wing cells) are present in mature corneas. As the cells continue to stratify upwards, they continue to flatten and form the superficial cell layers. Tight junctions, a crucial component of the corneal epithelial barrier function, are present in these layers (Hogan *et al.*, 1971). Unlike the epidermis, corneal epithelial cells do not lose their nucleus or undergo extensive keratinization. The process of terminal differentiation—leaving the basal cell layer, to sloughing from the superficial cell layer—takes approximately 7 days (Beebe and Masters, 1996; Hanna and O'Brien, 1960). Interestingly, it has recently been demonstrated (Norman *et al.*, 2004) that there may be genes that are differentially expressed in each layer.

Corneal stroma

The stroma plays a major role in all the main functions of the mature cornea: protection, transmittance of light, and refraction of light. All of these functions are related to the highly organized extracellular matrix. This matrix is formed primarily by aligned bundles of collagen termed lamellae, which are stacked in an orthogonal pattern (Hogan *et al.*, 1971).

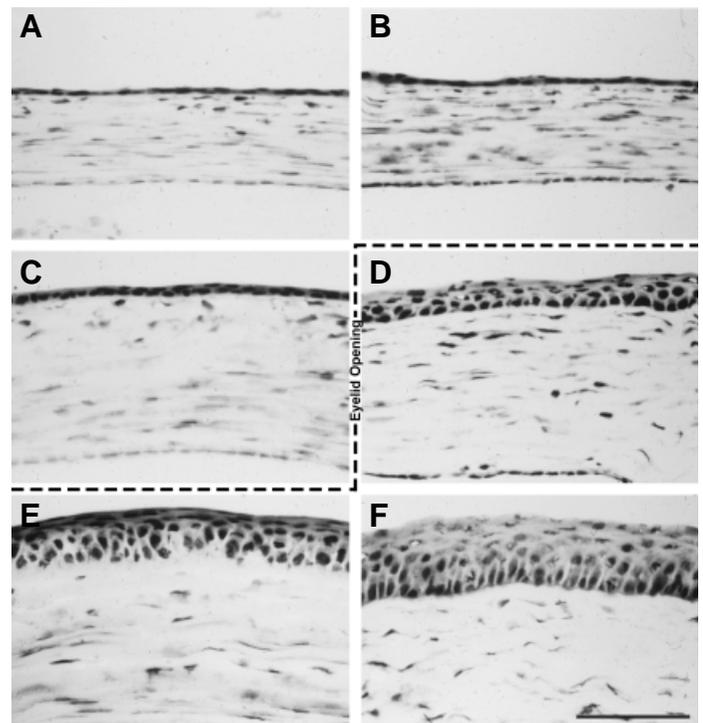As with the epithelium, the stroma also undergoes dramatic alter-

Fig. 2. Differentiation of central corneal epithelium associated with eyelid opening. *At one **(A)** and seven **(B)** days-of-age, the central corneal epithelium is composed of one or two layers of cells. At ten days **(C)**, just before eyelid opening, the number of cell layers increases to two or three. At two weeks **(D)**, just after eyelid opening, four-to-five layers are observed. At three weeks **(E)**, five-to-six layers are present, and at four weeks **(F)**, the epithelium is indistinguishable from that of an adult. Note the shape change of the basal cells from flat ovoid to columnar that occurs as the epithelium differentiates. Also note the increase in stromal thickness and decrease in stromal cell density that occurs between seven and ten days-of-age. Bar, 50 µm. (Reproduced from Chung et al. (Chung et al., 1992), courtesy of* Investigative Ophthalmology and Visual Science.*)*

ations in thickness and cellularity at the time of eyelid opening. As seen in Fig. 2 B,C, stromal thickness increases significantly just prior to eyelid opening, and the density of cells appears to decrease. Song *et al.* (Song *et al.*, 2003) examined this observation quantitatively in the developing mouse and found that the thickness of the stroma increased a remarkable 60% in the four days preceding eyelid opening. The same authors found that the density of stromal cells decreased by approximately 50% over the same time period. It is not known if the number of cells actually decreased or if the change in density was the result of the increase in stromal volume.

Corneal endothelium

The mature corneal endothelium functions to maintain the stroma in a relatively dehydrated state. This function depends on the presence of a barrier formed by focal tight junctions, which prevent fluid flow, and on a 'pumping' action provided by Na^+/K^+-ATPase and Mg^{++}-dependent bicarbonate enzymes present in the lateral membranes of the endothelial cells (Joyce, 2003).

In the developing corneal endothelium, morphological alterations associated with eyelid opening are less dramatic than those observed in the stroma or epithelium. The endothelium remains a monolayer throughout the entire development of the cornea and gradually thins with age. However, changes do occur during the time surrounding eyelid opening that likely have a profound effect on the endothelium becoming non-proliferative and initiating its barrier function (Bahn *et al.*, 1986; Cintron *et al.*, 1988; Haustein, 1983; Joyce *et al.*, 2002; Murphy *et al.*, 1984). Through electron microscopic studies, it has been demonstrated that, just before eyelid opening, intracellular spaces close and junctional complexes mature. In addition, Descemet's membrane has been found to be assembled into its mature form, during this period.

Proliferative changes associated with eyelid opening

Perhaps the most profound change to occur that is associated with eyelid opening is the dramatic alteration in levels of proliferation (Francesconi *et al.*, 2000; Hanna and O'Brien, 1961; Joyce *et al.*, 1998). As seen in Fig. 3A, at the time of birth, cell proliferation is present in the epithelium, stroma, and endothelium. Approximately 20% of the epithelial and stromal cells, and 12% of the endothelial cells, are actively progressing through the cell cycle (Fig. 3). By the time of eyelid opening, the number of cells actively proliferating in both the stroma and endothelium has decreased to nearly zero. This low level of proliferation will be maintained throughout the life of the cornea. In contrast, the level of proliferation increases noticeably in the corneal epithelium (Fig. 3) and peaks with almost 75% of the basal cells actively proliferating (Fig. 3B). By three weeks-of-age, the number of proliferating cells drops to that seen in the adult (Figs. 3C, 4). This burst of proliferation correlates well time-wise with the stratification of the epithelium (Fig. 2).

Corneal epithelium

During the time between birth and eyelid opening, the number of proliferating cells in the corneal epithelium increases almost four-fold (Fig. 4). One of the potential mediators of this increase is signaling through the epidermal growth factor (EGF) receptor (Zieske and Wasson, 1993). As seen in Fig. 5 A-D, EGF receptor
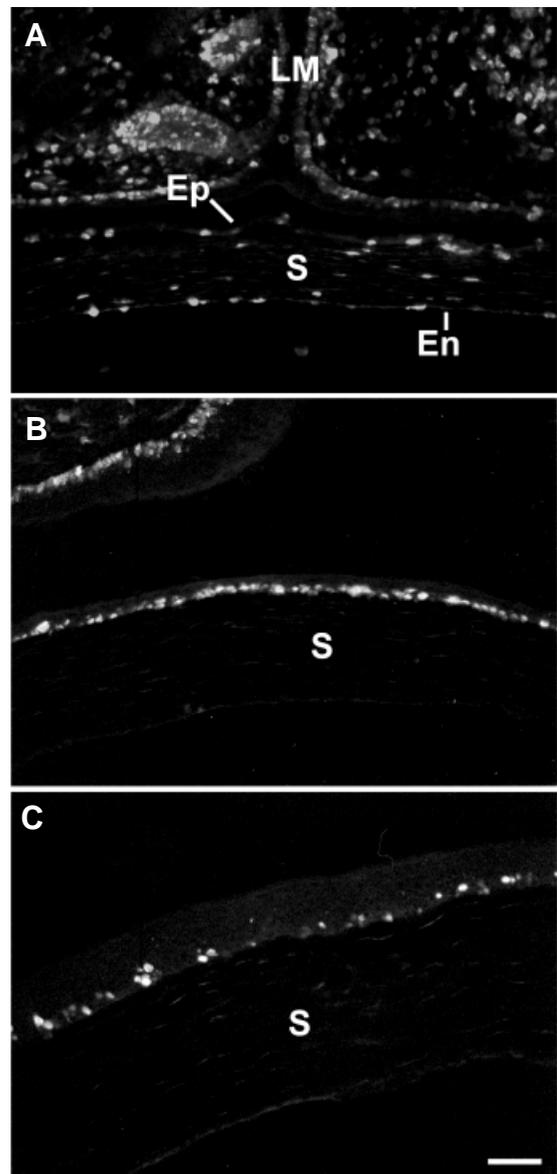

Fig. 3. Immunolocalization of Ki67 in developing rat cornea. *Ki67 (a marker of proliferating cells) was localized in central cornea of three-day-old* **(A)**, *fifteen-day-old* **(B)**, *and adult* **(C)** *rats. Ki67 was detected in the basal cell layer of corneal epithelium at all ages. Maximal localization of Ki67 in corneal epithelium correlated with eyelid opening (B). Note the dramatic decrease in proliferating cells in the stroma and endothelium that occurs between three days after birth (A) and one day after eyelid opening (B). LM, eyelid margin; Ep, corneal epithelium; S, stroma; En, endothelium. Bar, 50 μm. (Reproduced from Francesconi* et al. *(Francesconi et al., 2000), courtesy of* Investigative Ophthalmology and Visual Science.*)*

is present across the entire cornea at birth, and appears to increase at seven days—just prior to the increase in proliferative rate. This also correlates with the increase of EGF present in the plasma and presumably in the tear film (Beardmore and Richards, 1983; Thulesen *et al.*, 1994; Tsutsumi *et al.*, 1987). As the epithelium stratifies, the level of EGF receptor appears to decrease (Fig. 5 E,F). This could potentially lead to the cessation of the proliferative burst preventing hyper-stratification of the epithelium. The concept
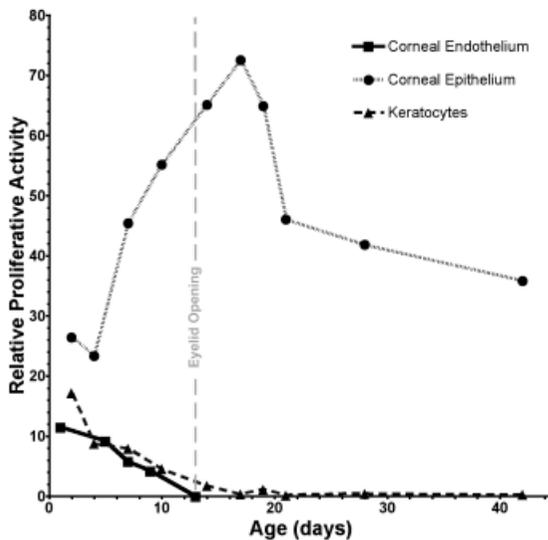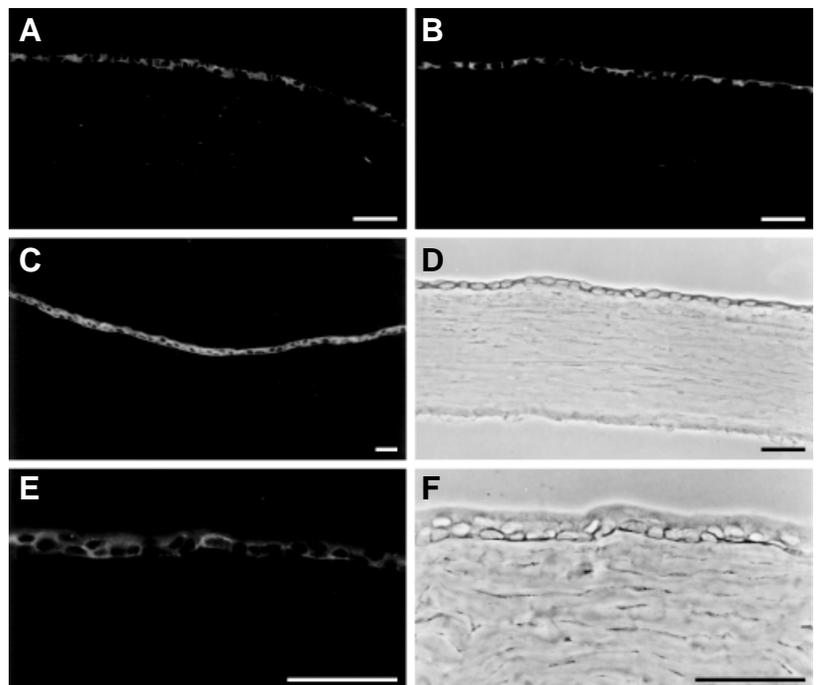
Fig. 4. Quantitation of proliferative cells during development. *The number of Ki67-positive cells in corneal epithelium and stromal keratocytes, and the number of bromodeoxyuridine-positive cells in the endothelium were quantified, as described in Francesconi* et al. *(Francesconi* et al., *2000) and Joyce* et al. *(Joyce* et al., *1998), respectively. (Adapted from Francesconi* et al. *(Francesconi* et al., *2000) and Joyce* et al. *(Joyce* et al., *1998), courtesy of* Investigative Ophthalmology and Visual Science.*)*

that signaling through the EGF receptor family is involved in epithelial stratification is supported by the findings of Xie *et al.* (Xie *et al.*, 1999). They found in transgenic mice that were overexpressing the EGF receptor, ErbB2, that the mice were born with 5-7 layers of epithelial cells instead of the 2 layers seen in wild-type controls. Interestingly, the keratocytes also appeared to be hyperplastic, and the collagen lamellae were not arranged as precisely as seen in control mice.

Several proteins associated with cell cycle regulation have been examined during corneal development. Among these are the three members of the retinoblastoma family, as well as the E2F family of transcription factors (Francesconi *et al.*, 2000). The retinoblastoma gene product (pRb) is one of the key regulators of the cell cycle, and is normally hypophosphorylated in quiescent cells. When quiescent cells are stimulated to proliferate, pRb is phosphorylated, thereby disrupting the interaction between pRb and E2F. Free E2F is then responsible for activating the transcription of genes necessary for DNA synthesis. In the developing corneal epithelium, pRb is present in the nuclei of all cells at all time points, and its expression and localization remain

constant (Francesconi *et al.*, 2000). We are aware of no evidence that phosphorylation of pRb is involved in corneal epithelial stratification. In contrast to pRb, the expression and localization of the other two members of the retinoblastoma family, p107 and p130, are altered during corneal development.

At birth, p107 exhibits a nuclear localization in the basal cells across the cornea. During the period between birth and eyelid opening, p107 is increasingly localized in the cytoplasm, until little, if any, nuclear localization is observed. The significance of this change in localization is not clear. Interestingly, nuclear localization is maintained in the limbal basal cells.

The final member of the family, p130, differs from pRb and p107 in that it is phosphorylated in quiescent cells and its levels decrease in proliferating cells (Francesconi *et al.*, 2000). Thus, its levels have been shown, in other systems, to be high in quiescent and terminally differentiated cells. In the rat corneal epithelium, p130 is not present at appreciable levels until stratification occurs. At approximately nine days-of-age, p130 is first observed in the nuclei of the non-basal, non-proliferating cells. Little, if any, p130 is localized in the proliferative basal epithelial cell layer. As the epithelium develops, the localization is confined to the terminally differentiated suprabasal cell layers. This pattern is maintained in the adult and is consistent with p130 playing a role in corneal epithelial differentiation (Francesconi *et al.*, 2000).

Among the E2F family, only E2F1 localization changes during development (Francesconi *et al.*, 2000). E2F1 is initially present at low levels until just before eyelid opening, when a nuclear localization is seen. This pattern is maintained in the adult and is consistent with E2F1 being a marker of differentiation; however, it is not clear what genes E2F1 may be regulating.

Corneal stroma
During the period between birth and eyelid opening in the rat, the number of proliferating cells decreases from approximately 20% of

Fig. 5. Immunolocalization of EGF receptor in developing rat corneas. *EGF receptor is localized at high levels in rat corneal epithelium at one **(A)**, four **(B)**, and seven **(C)** days-of-age. **(D)** Phase-contrast micrograph of the area shown in (B). Note that only one or two layers of epithelial cells are present. As the epithelium begins to stratify, the level of EGF receptor appears to diminish **(E)**; central cornea, ten days of age. **(F)** Phase-contrast micrograph of the area shown in (E). Note that a multilayered epithelium is present. Bars, 50 μm. (Reproduced from Zieske and Wasson (Zieske and Wasson, 1993), courtesy of* Journal of Cell Science.*)*

the total to essentially zero (Fig. 4). The mechanism for this decrease is unknown. Only a few studies have examined proteins associated with cell proliferation in corneal keratocytes during development. pRb is present in the nucleus of all stromal cells at all ages. p107 is present in low levels, until approximately three weeks-of-age and then decreases to undetectable levels. Most interestingly, p130 does not appear to be present until nine days-of-age, when a nuclear localization is observed. Its levels appear to increase as the proliferative rate of the keratocytes decreases and the cells become quiescent (Francesconi *et al.*, 2000). In addition, it has been observed that, in the adult, the keratocytes express very little, if any, cyclins or cyclin-dependent kinase inhibitors (Joyce *et al.*, 1996). This suggests that, during the time of eyelid opening, the keratocytes have exited the cell cycle and become quiescent G0 cells. The fact that the cells are in G0 rather than being terminally differentiated is supported by the findings that stromal keratocytes can be readily stimulated to proliferate in response to corneal wounding (Zieske *et al.*, 2001).

Corneal endothelium

At birth, as many as 12% of corneal endothelial cells are actively undergoing cellular proliferation (Fig. 4). By the time of eyelid opening, the rate of proliferation drops to zero. This change in proliferative rate has been associated with the contact inhibition that occurs during the period prior to eyelid opening. Joyce and coworkers (Joyce *et al.*, 2002) found that several proteins present in cell-cell junctions, including ZO-1, connexin 43, and Na^+/K^+-ATPase, are expressed in corneal endothelium at birth, in the rat, but they do not localize into mature junctions until just prior to eyelid opening. Contact inhibition of cell proliferation appears to be mediated through the cell cycle inhibitor $p27^{KIP1}$. Several lines of evidence support this conclusion including: (1) $p27^{KIP1}$ levels are reduced when endothelial cells are stimulated by fibroblast growth factor-2 (Lee and Kay, 2003); (2) SV-40-transformed endothelial cells express less $p27^{KIP1}$ than untransformed cells (Schonthal *et al.*, 1999); and (3) rat endothelial cells upregulate $p27^{KIP1}$ expression 20 fold when they become contact inhibited (Joyce *et al.*, 2002).

In contrast to corneal keratocytes, corneal endothelial cells do not appear to enter the G0 phase of the cell cycle after eyelid opening; rather, they appear to be arrested in the G1 phase of the cycle. This finding is based on the observation that the endothelial cells continue to express G1-phase-associated proteins, such as cyclins D and E, after eyelid opening and throughout adulthood (Joyce *et al.*, 1996). Since $p27^{KIP1}$ is expressed in the adult cells, it appears that this inhibitor plays a major role in maintaining the cell cycle arrest (Joyce *et al.*, 1998). It is an intriguing but unanswered question—why do the corneal keratocytes and endothelial cells use two separate mechanisms to achieve quiescence? The two cell types also vary widely in their ability to re-enter the cell cycle, in that endothelial cells in rat, mouse, and human corneas cannot be readily stimulated to proliferate.

Alterations in gene expression associated with eyelid opening

As seen in Table 1, a number of gene products have been observed to be either up- or downregulated during the period of eyelid opening. These proteins can be roughly separated into four

groups: (1) epithelial differentiation markers, (2) tear-film-associated proteins, (3) matrix components, and (4) cell-cycle-associated proteins. These proteins give clues to the important development processes that are altered around the time of eyelid opening. A recent report by Norman *et al.* (Norman *et al.*, 2004) dramatically expanded this list. These authors used serial analysis of gene expression (SAGE) to compare adult and nine-day-old mouse cornea. Their findings suggest that only one-third of the expressed genes were common between the two groups, spectacularly supporting the concept that eyelid opening correlates with a major developmental step in the cornea.

Epithelial differentiation markers

Several proteins associated with epithelial differentiation have been observed to be either up- or downregulated in conjunction with eyelid opening. The expression and localization of these proteins can be roughly grouped into two categories. In the first group of proteins, which include α-enolase (Chung *et al.*, 1992), α9 integrin (Stepp *et al.*, 1995), and EGF receptor (Zieske and Wasson, 1993), expression is seen in cells across the cornea at the time of birth (Figs. 6 and 7). During the period between birth and
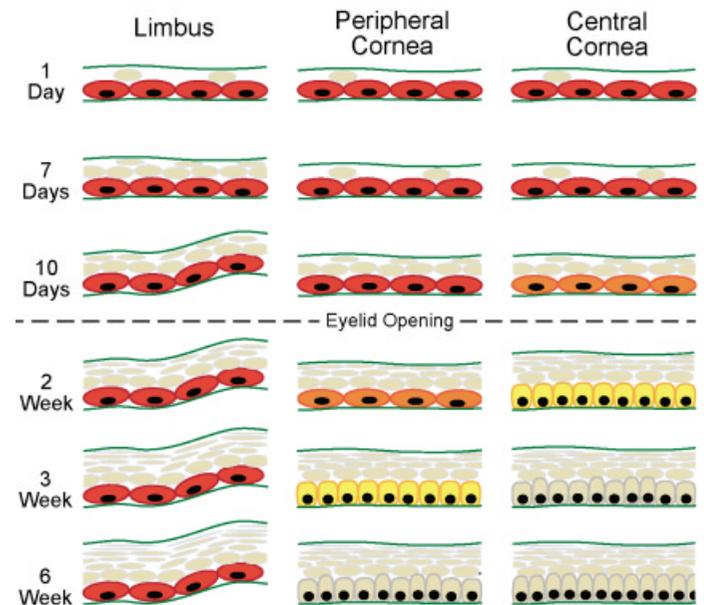

Fig. 6. Diagrammatic summary of changes in stratification, cell shape and expression of α-enolase during corneal development. *All areas of the corneal epithelium, including central cornea, peripheral cornea, and limbus, expressed α-enolase at high levels until ten days-of-age. At two weeks, change of cellular shape, epithelial stratification, and decreased expression of α-enolase occurred at the central cornea, and basal cell shape changed from ovoid to cuboidal. Increased stratification of four-to-five layers of cells was seen concomitantly. At three weeks, basal cells in the peripheral cornea showed similar changes to those seen in the central cornea at two weeks. Also at three weeks, basal cells in the central cornea no longer expressed α-enolase and began to change in shape from cuboidal to columnar. Basal cells in the limbus never changed their level of expression of α-enolase or their ovoid shape. Note: the intensity of the shading in the basal cells indicates the level of expression of α-enolase. (Adapted from Chung* et al. *(Chung* et al.*, 1992) and reproduced courtesy of* Investigative Ophthalmology and Visual Science.*)*
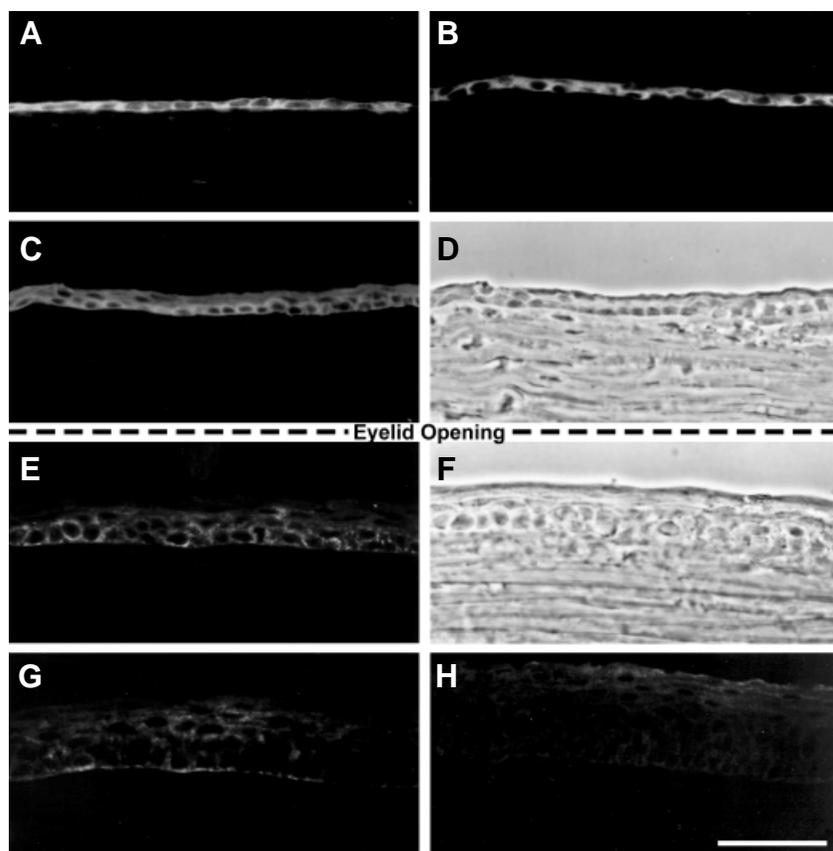
Fig. 7. Localization of α-enolase in central cornea of developing rats. *α-Enolase was expressed at high levels in all basal cells in the corneal epithelium at one* **(A)** *and seven* **(B)** *days-of-age. At ten days* **(C)**, *expression decreased in central cornea;* **(D)** *phase contrast of area shown in (C). At two weeks* **(E)**, *expression was greatly reduced and cellular shape changed to cuboidal;* **(F)** *phase contrast of the area shown in (E). Basal cells in the central cornea no longer expressed α-enolase after three weeks* **(G)***; six weeks* **(H)**. *Bar, 50 μm. (Reproduced from Chung et al. (Chung et al., 1992), courtesy of Investigative Ophthalmology and Visual Science.)*

eyelid opening, the expression of these proteins decreases in the central cornea and becomes increasingly restricted to the basal cell layer of the limbus. Within one-to-two weeks after eyelid opening, these proteins are localized in a pattern that they will maintain throughout adulthood. Furthermore, since they are concentrated in the limbal basal cell layer, which houses the corneal epithelial stem cells, they have been postulated to be markers of the corneal stem cells (Schermer *et al.*, 1986; see Wolosin *et al.*, (2004), for a more complete discussion of corneal stem cells.) It is not clear if these proteins are actual stem cell markers, but their localization pattern has lead to the hypothesis that, prior to eyelid opening, the epithelial cells in central cornea are stem cell-like, and that this property is lost in association with eyelid opening (Chung *et al.*, 1992; Stepp *et al.*, 1995; Zieske and Wasson, 1993).

In the second group of proteins, which includes keratins 3 (Chaloin-Dufau *et al.*, 1990; Rodrigues *et al.*, 1987) and 12 (Chaloin-Dufau *et al.*, 1990; Liu *et al.*, 1993), transketolase (Sax *et al.*, 2000), connexin 43 (Wolosin *et al.*, 2002), and aldehyde dehydrogenase (Burton *et al.*, 1999; Downes and Holmes, 1992; Kays and Piatigorsky, 1997), increased expression has been

observed in conjunction with eyelid opening. These proteins exhibit a reciprocal localization with those in the first group, in that they are present at low levels at birth and increase as the epithelium stratifies and matures. Interestingly, these proteins are not expressed in the basal cell layer of the limbus. This finding has lead to the hypothesis that the limbal basal cells are the least-differentiated cells in the corneal epithelium, and that this cell layer comprises the stem cell population (Schermer *et al.*, 1986). An intriguing but unanswered question is whether the same regulatory factors control these proteins.

Tear-film-associated proteins

A second major grouping of proteins that are induced during the period corresponding to eyelid opening are proteins that are either components of the tear film or associated with maintaining the tear film. Several of these proteins have been identified: mucin 4 (muc4) (Tei *et al.*, 1999), α 2,3-sialyltransferase (Uehara *et al.*, 1995), and an ocular surface glycocalyx component (Watanabe *et al.*, 1993) that may be muc16 (Argueso *et al.*, 2003). In contrast to proteins that increase in expression as the epithelium differentiates (see above), the expression of the tear-film-associated mucins, muc4 and muc16, closely correlated with eyelid opening (Tei *et al.*, 1999; Watanabe *et al.*, 1993). Indeed, Watanabe *et al.* (Watanabe *et al.*, 1993) observed that muc16 was not detected until one day after eyelid opening. These authors also observed that, in cases of asynchronous eyelid opening, the open eye expressed the glycocalyx component, while the closed eye did not, and that surgically opening the closed eyelid led to rapid expression of the tear-film-associated mucins. This report suggests that eyelid opening stimulates the expression of tear-film-associated proteins. However, it is not clear if the stimulus is light, oxygen, or another unknown stimulus.

Matrix components

The period between birth and eyelid opening has been observed to involve major changes in the architecture of the stroma in rabbit, mouse, and rat corneas (Fig. 1) (Chung *et al.*, 1992; Cintron *et al.*, 1983; Song *et al.*, 2003). These changes are manifested as a swelling of the stroma prior to eyelid opening and a thinning after opening. This alteration is accompanied by a decrease in stromal cell density. The swelling and subsequent thinning appears to be related to the sulfation of the proteoglycan portion of the lumican—a major keratan sulfate proteoglycan of the cornea (Song *et al.*, 2003). Sulfation occurs around the time of eyelid opening in the mouse and just subsequent to opening in the rabbit (Blochberger, Cornuet *et al.*, 1992; Blochberger, Vergnes *et al.*, 1992; Smelser and Ozanics, 1957; Song *et al.*, 2003; Ying *et al.*, 1997). Presumably, the synthesis of keratan sulfate leads to an increase in water absorption and consequent swelling of the cornea. Subsequently, the maturation of the endothelial cell layer leads to the pumping of water from the stroma and consequent

TABLE 1

PROTEINS ALTERED IN ASSOCIATION WITH EYELID OPENING

Proteins	References
Epithelial differentiation markers	
Aldehyde dehydrogenase (class 3)	(Burton *et al.*, 1999; Downes and Holmes, 1992; Kays and Piatigorsky, 1997)
α-enolase	(Chung *et al.*, 1992)
α9 integrin	(Stepp *et al.*, 1995)
Connexin 43	(Wolosin *et al.*, 2002)
Epidermal growth factor receptor	(Zieske and Wasson, 1993)
Keratin 3	(Chaloin-Dufau *et al.*, 1990; Rodrigues *et al.*, 1987)
Keratin 12	(Chaloin-Dufau *et al.*, 1990; Liu *et al.*, 1993)
Transketolase	(Sax *et al.*, 2000)
Tear-film-associated proteins	
α 2,3-sialyltransferase	(Uehara *et al.*, 1995)
Mucin 4	(Tei *et al.*, 1999)
Rat ocular surface glycocalyx protein	(Watanabe *et al.*, 1993)
Matrix components	
Keratan sulfate proteoglycan	(Blochberger, Cornuet *et al.*, 1992; Blochberger, Vergnes *et al.*, 1992; Smelser and Ozanics, 1957; Song *et al.*, 2003; Ying *et al.*, 1997)
Lumican	(Song *et al.*, 2003)
Tenascin C	(Maseruka *et al.*, 2000)
Type XII collagen (long form)	(Anderson *et al.*, 2000)
Cell cycle associated proteins	
E2F1	(Francesconi *et al.*, 2000)
p27	(Joyce *et al.*, 1998)
p107	(Francesconi *et al.*, 2000)
p130	(Francesconi *et al.*, 2000)
Others	
Glutathione peroxidase 1	(Fujii *et al.*, 2003)
Calcitonin gene-related peptide	(Jones and Marfurt, 1991)

thinning of the cornea. This process appears to be important in the development of corneal clarity. Studies of lumican-deficient mice support the importance of this proteoglycan (Song *et al.*, 2003). In these mice, the cornea does not swell around the time of eyelid opening, and the corneas have an elevated level of light scattering. Other matrix materials are also altered around the time of eyelid opening, but the role of these components is less clear.

Cell-cycle-associated proteins

As discussed in earlier sections of this review, major changes in the proliferative rate of cells in all three layers of the cornea occur around the time of eyelid opening. These changes include increased proliferation of the epithelium and decreased proliferation of the endothelium and stroma. The proliferative rates appear to correlate with alterations in the expression of cell cycle inhibitors, members of the retinoblastoma family, and members of the E2F family of transcription factors. The increase in epithelial proliferation corresponds with the increased level of stratification that occurs during this period. It is usually assumed that decreased proliferation of the endothelial cell layer is necessary to maintain a monolayer of cells that provide optimal fluid transport. It is less clear why the stromal cells become non-proliferative, but one explanation is that this allows the architecture of the stroma to remain stable - enabling the tissue to maintain transparency.

Regulation of gene expression associated with eyelid opening

Although an increasing amount of information is available about the events associated with eyelid opening, as well as alterations in gene expression, the regulation of these events is still poorly understood. Several recent articles have used molecular biological techniques to examine factors potentially involved in corneal development. Certainly, one of the key regulators of corneal and eye development is the transcription factor Pax6—mice lacking Pax6 do not form eyes. Davis *et al.* (Davis *et al.*, 2003) have also demonstrated that Pax6 plays an essential role in corneal development by examining the Pax6 heterogeneous SEY mouse. They found that the corneal epithelium did not stratify to its normal thickness in these mice, and that cell-cell, as well as, cell-matrix adherence was compromised. Their data suggest that Pax6 is necessary for epithelial stratification; however, it is unclear if an environmental cue is required to stimulate the action of Pax6 in association with eyelid opening. Other factors that have been demonstrated to be involved in corneal development are TGF-β1 (Flugel-Koch *et al.*, 2002), notch1 (Nicolas *et al.*, 2003), TGF-α (Reneker *et al.*, 2000), the forkhead/winged-helix gene (Kidson *et al.*, 1999), and IκB kinase α (Yoshida *et al.*, 2000). Intriguingly, both overexpression of TGF-α and deletion of forkhead lead to the lack of an endothelial cell layer. However, in the first case, the epithelial layer is thinned, while in the second, the epithelium is thickened.

In addition to molecular factors, it is likely that alteration in the cornea's environment during eyelid opening plays a role in differential gene expression. Potential environmental cues include changes in light and oxygen. To examine the potential role of light in the expression of the epithelial differentiation marker transketolase, Sax *et al.* (Sax *et al.*, 2000) compared mice reared in the dark to their normal littermates. They observed that the level of transketolase mRNA was two-fold lower in the dark-raised mice, indicating that light was a stimulus. However, the dark-reared mice still exhibited a significant increase in transketolase message levels upon eyelid opening, indicating that light was not the only cue. This is supported by findings in human corneas, which open before birth. These corneas exhibit the same type of changes in epithelial differentiation and expression of differentiation markers as in species where the eyelids open after birth (Rodrigues *et al.*, 1987). In another transgenic mouse study, Saika *et al.* (Saika *et al.*, 2001) found that TGFβ-2 knockout mice had a decreased accumulation of extracellular matrix materials and developed a thin cornea. Thus, it appears that TGFβ-2 is an important signaling molecule, but its stimulus remains unknown.

In summary, the development of the cornea associated with eyelid opening involves many intriguing processes that are vital for the maturation of a light transparent cornea; however, many questions remain about how these processes are regulated. Several molecular factors have been identified that play a role in corneal development; however, their role during eyelid opening and the effect of environmental cues still await elucidation.

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