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Positive influence of AP-2 α transcription factor on cadherin gene expression and differentiation of the ocular surface

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Abstract

The family of transcription factors Activating protein-2 (AP-2) are known to play important roles in numerous developmental events, including those associated with differentiation of stratified epithelia. However, to date, the influence of the AP-2 genes on endogenous gene expression in the stratified epithelia and how this affects differentiation has not been well defined. The following study examines the detailed expression of the AP-2 α and AP-2 β proteins in the stratified epithelia of the ocular surface, including that in the cornea and developing eyelids. The effect of altered levels of the AP-2 α gene on ocular surface differentiation was also examined using a corneal epithelial cell line and AP-2 α chimeric mice. Immunolocalization studies revealed that, while AP-2 β was broadly expressed throughout all cell layers of the stratified corneal epithelium, AP-2 α expression was confined to cell compartments more basally located. AP-2 α was also highly expressed in the less differentiated cell layers of the eyelid epidermis. Overexpression of the AP-2 α gene in the corneal cell line, SIRC, resulted in a dramatic change in cell phenotype including a clumping growth behavior that was distinct from the smooth monolayer of the parent cell line. Accompanying this change was

an up-regulation in levels of the cell adhesion molecule, N-cadherin. Examination of the ocular surface of AP-2 α chimeric mice, derived from a mixed population of AP-2 $\alpha^{-/-}$ and AP-2 $\alpha^{+/+}$, revealed that a down-regulation in E-cadherin expression is correlated with location of the AP-2 $\alpha^{-/-}$ null cells. Together, these findings demonstrate that AP-2 α participates in regulating differentiation of the ocular surface through induction in cadherin expression.

Keywords

ocular surface; cell adhesion; transcription factors; AP-2; cornea; eyelids; differentiation

Introduction

Development and regeneration of stratified squamous epithelia, such as the epidermis of the skin and the corneal epithelium of the eye, involve complex regulatory mechanisms for cell determination and differentiation. In these tissues, the daughter cells, formed from the division of transient amplifying cells, leave the basal cell layer and migrate progressively upward toward the epithelial surface. With each step into a new epithelial layer, cells acquire differentiated characteristics specific to that layer. As they reach the top, cells die and are gradually shed from the surface. The order of synthesis of many structural proteins in this program of differentiation, including cytoskeletal proteins like the keratins and cell adhesion molecules such as integrins and cadherins, are well characterized (Nelson and Sun, 1983; Tseng et al., 1984; Schermer et al., 1989; Stepp et al., 1990; Fuchs and Byrne, 1994). However, the nature and specific role(s) of the regulatory molecules governing the activation or repression of these genes during epithelial differentiation are largely unknown.

Activating protein-2 (AP-2), consists of a family of developmentally important transcription factors which are thought to regulate genes involved in a number of complex biological events, including those associated with differentiation of the epidermis and corneal epithelium (Fini et al., 1994; Fuchs and Byrne, 1994; Gille et al., 1997; West-Mays et al., 1999).

Putative DNA binding sites for AP-2 exist in the 5' upstream region of many epithelial-specific genes and AP-2, and related factors have also been shown to regulate the transcriptional promoter activity of epithelial genes transfected into cultured cells including intermediate filaments, such as the keratins and cell adhesion molecules like the cadherins and integrins (Snape et al., 1990; 1991; Behrens et al., 1991; Fuchs and Byrne, 1994; Sinha et al., 2000). Likewise, in cultured corneal epithelial cells, AP-2 has been shown to regulate the transcription of gene promoters involved in differentiation such as the K3 keratin gene (Chen et al., 1997) and gelatinase B, a matrix degrading metalloproteinase involved in corneal epithelial repair (Fini et al., 1994). The majority of this work, however, has focused on artificially created gene promoter constructs. The *in vivo* downstream targets of the AP-2 genes and how they affect epithelial differentiation are not fully understood.

To date, four separate, homologous AP-2 genes (α , β , γ and δ) have been identified (Williams and Tjian, 1991; Moser et al., 1995; Chazaud et al., 1996; Zhao et al., 2001) and exhibit both overlapping as well as distinct expression patterns in the developing murine embryo (Moser et al., 1997b). In the developing and adult mouse eye, we have shown in previous studies that both AP-2 α and AP-2 β proteins have dynamic expression patterns. Both the AP-2 α and β proteins are expressed in the embryonic and post-natal corneal epithelium (West-Mays et al., 1999). *In situ* hybridization studies have also indicated the presence of a strong signal for AP-2 β mRNA in the embryonic cornea and a signal for AP-2 α in the developing eyelids (Moser et al., 1997b).

Unique developmental phenotypes occur in mice in which either the AP-2 α or β gene has been deleted (Nottoli et al., 1998). AP-2 α null mutants exhibit severe craniofacial defects including neural tube defects and exencephaly, as well as multiple ocular defects (Schorle et al., 1996; Zhang et al., 1996; West-Mays et al., 1999). In contrast, AP-2 β null mutants exhibit polycystic kidney disease attributed to enhanced apoptotic cell death (Moser et al., 1997a) and do not have overt eye defects. Interestingly, neither the AP-2 α nor the AP-2 β mutants exhibit epidermal defects despite the high expression of these factors in the wild-type epidermis and evidence for AP-2 in controlling epidermal gene expression. One possible explanation is that the AP-2 γ gene, which is also expressed in the developing epidermis, may play a role. However, it is also plausible that the AP-2 α and AP-2 β genes, which have a high homology, exhibit redundancy and/or compensation in their function(s), at least in the epidermis. Further investigation of the role(s) of AP-2 α and AP-2 β in differentiation of the ocular surface tissues using the mouse mutants is hampered by either earlier developmental defects or lethality at birth, events which precede differentiation of the ocular surface (Moser et al., 1997a; West-Mays et al., 1999). Thus, an *in vivo* role for the AP-2 genes in development and differentiation of multiple stratified epithelial tissues remains unclear.

In the following study, we further explore the role(s) of the AP-2 proteins in differentiation of the ocular surface, including the stratified epithelium of the cornea and that of the developing eyelids. The differential expression pattern of AP-2 α and AP-2 β proteins in the developing ocular surface was examined and the effect of altering AP-2 α expression on differentiation was investigated using a corneal epithelial cell line and AP-2 α chimeric mice. These data demonstrate an *in vivo* regulatory role for AP-2 α in ocular surface differentiation.

Methods

Immunohistochemistry

Animal procedures were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Embryonic mice (E18 and P1) were collected and staged as reported previously (West-Mays et al., 1999). Embryos and adult mouse eyes were embedded in Tissue TekII OCT Compound (Labtech products Naperville, IL), frozen in liquid nitrogen, and sectioned at 5 and 7 μ m. Indirect immunofluorescence was then used to detect AP-2 and E-cadherin proteins on the sections. Sections were air dried for one hour and then fixed in acetone (10 min) on ice. The sections were blocked with normal serum for 20 minutes, followed by incubation with one of the following primary antibodies: A commercially available polyclonal AP-2 α antibody (SC-8975; Santa Cruz Biotechnology Inc., Santa Cruz, CA) which recognizes an AP-2 α specific epitope in the mouse was applied to sections (1:100; at room temperature). A rabbit polyclonal antibody was also used which recognizes AP-2 β (Bosher et al., 1996) (1:3500; 1 hour incubation at room temperature). Following incubation with one of the primary antibodies, either a FITC- or Rhodamine-conjugated secondary antibody (Chemicon, Temecula, CA) was applied for 1 hour at 1:300 (room temperature). Indirect immunohistochemistry was used to detect expression of E-cadherin and N-cadherin on the sections from AP-2 α chimeric mice. Two different monoclonal N-cadherin antibodies were used (Knudsen et al., 1995 or Becton Dickinson, San Jose, CA; #550038) either neat or at a 1:10 dilution. Additional antibodies used include those specific for E-cadherin at 1:250 (Signal Transduction Labs, Lexington, KY; Zymed Laboratories Inc., San Francisco, CA). The cadherin antibodies were separately incubated on frozen sections, and the location of each endogenous protein was revealed using an indirect Biotin/Avidin-Immunoperoxidase system (Vector Laboratories, Burlingame, CA). For each experiment, a section was stained without primary antibody to serve as a negative control. Immunofluorescent and immunoperoxidase staining was visualized with a Nikon Eclipse E400 microscope, and images were captured with

a high-resolution color digital camera (Spot; Diagnostics Instruments COO Color digital) and reproduced for publication using Adobe Photoshop 6.0 (Adobe System Inc.).

Generation of AP-2 α chimeric mice

AP-2 α chimeric mice were generated as previously described (Nottoli et al., 1998; West-Mays et al., 1999). Briefly, AP-2 α $-/-$ embryonic stem (ES) cells were generated from the embryonic stem cell line containing a disruption of one allele of the AP-2 α gene (Zhang et al., 1996). A *LacZ* gene was inserted into the remaining wild-type allele of the AP-2 α gene to produce a similar mutation and disrupt AP-2 α function. The ES cells were then microinjected into 3.5 dpc (days post-conception) C57BL/6 blastocysts using standard techniques. Injected embryos were then transferred into pseudopregnant SW females, and mice were either allowed to go to term or sacrificed at E18. Following dissection, the embryos were fixed in 0.2% paraformaldehyde and embedded for frozen sectioning in O.C.T. medium. Sections were cut at 7 μ m and either stained for β -galactosidase (*LacZ*) activity (West-Mays et al., 1999), in order to determine location of cell progeny, or stained with either the AP-2 α antibody or the cadherin antibodies according to the procedures discussed above.

Stable transfection of cells

The rabbit corneal epithelial-derived cell line SIRC was cultured as recommended by the supplier (American Type Culture Collection, Rockville, MD). SIRC cells were then transfected with an AP-2 α cDNA that was cloned by PCR using the human AP-2 α sequence (Williams and Tjian, 1991). Transfection was performed using lipofectamine (Gibco BRL) and the stable transformation approach. In preparation for transfection, the wild type AP-2 α gene was recloned into the expression vector pcDNA3 (Invitrogen), containing a gene for neomycin resistance which allows for selection of cells containing the construct stably integrated into their genome. Cells were plated for transfection as described earlier (Fini et al., 1994) and subsequently grown in geneticin (Gibco BRL 400 μ g/ml) for neomycin resistance to obtain clones. Colonies of cells derived from a single cell were then selected and amplified. Some of the SIRC cells were also transfected just with the vector pcDNA3, and these were considered the parent control cells.

Adhesion assay

To test the ability of the SIRC clones to adhere to a variety of extracellular matrices, a cytoMatrix (ECM200; Chemicon) adhesion assay kit was used. Cells were plated as recommended by the manufacturer into 96-well culture dishes previously coated with optimized concentrations of human extracellular matrix proteins, including fibronectin, vitronectin, laminin, collagen type I, collagen type IV, or tenascin. The cells were allowed to adhere, and after washing away the non-bound cells, the binding of cells was semi-quantitated by staining with 0.2% crystal violet and reading the wells on a microtiter plate. Note that the higher the OD the better the adhesion. Clones, as well as control cells, were assayed for each matrix in 6 replicates. Statistically significant differences were determined by use of the Student's *t*-test. A value of $P < 0.05$ was considered significant.

Western and Northern blotting

Whole cell lysates of untransfected and stably transfected SIRC cells were prepared for Western blot analysis as outlined by the manufacturer of the antibodies used (Santa Cruz). Protein concentration was measured by Bio-Rad dye binding assay (Bio-Rad, Hercules, CA). Aliquots of equal protein were frozen at -70°C and thawed just prior to use. Equal amounts of protein were separated by SDS-PAGE and transferred to Immobilon P membrane (Millipore, Bedford, MA). Blots were either probed with AP-2 (SC-184) antibody (1:250) alone or simultaneously probed with AP-2 (SC-184) (1:250) antibody and a monoclonal N-cadherin

antibody (Knudsen et al., 1995) (1:300). Additional antibodies used include those specific for E-cadherin (Signal Transduction Labs, Lexington, KY; Zymed Laboratories Inc., San Francisco, CA) and Pan-cadherin (Nelson et al., 1990). Incubation times were 1 hour at room temperature. Following these incubations, blots were probed with an HRP-conjugated secondary and ECL detection reagents as recommended by the manufacturer (Amersham, Arlington Heights, IL). After film exposure, blots were stained with 0.1% amido black to confirm the equal loading of total proteins between lanes. Densitometry was performed on autorads using an Eagle Eye System (Stratagene, La Jolla, CA) to determine the fold induction of N-cadherin and AP-2 protein isolated from SIRC clones relative to controls.

For Northern blot analysis, total RNA was isolated from control and transfected SIRC cells. A human cDNA probe for AP-2 was labeled with ^{32}P by random priming. Loading equivalence between gel lanes was ascertained by probing for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) message with a human cDNA (Allen et al., 1987). Densitometry was performed on autorads using an Eagle Eye System (Stratagene, La Jolla, CA) to determine the fold induction of AP-2 α mRNA in the SIRC clones relative to controls. These levels were normalized to the levels of GAPDH.

Results

Expression of AP-2 α and AP-2 β in the developing ocular surface

To investigate whether the AP-2 α and AP-2 β proteins become differentially expressed as the ocular surface develops, immunolocalization was performed on adjacent sections of whole embryos (E12.5 and P1) or corneas from adult (>4 week) mice using antibodies specific for each protein, AP-2 α and AP-2 β . Since in a previous study we had outlined the expression pattern for the AP-2 proteins in the developing lens and retina, and to some degree, the cornea, our results here will focus on the developing eyelids, conjunctiva, and further details of the corneal epithelium. At E12.5, distinct AP-2 α expression was found in the epithelial surface tissue adjacent to the eye region which later forms the eyelid epidermis and the conjunctiva (Fig. 1A). As previously reported, AP-2 α and AP-2 β expression were also detected in the single layer of cuboidal epithelium forming the corneal epithelium (data not shown). At P1, AP-2 α expression was observed in the eyelid epidermis with specific, intense staining in the nuclei of the more basally located cells of the epidermal surface (Fig. 1B). Less AP-2 α staining was observed in the more suprabasal, stratum granulosum layer, and no staining was observed in the superficial keratinized layers of the developing eyelids. Strong nuclear staining for AP-2 α was also detected in the immature hair follicles just beneath the epidermal layer (Fig. 1B). In the junctional epithelium, where the eyelids remain fused, AP-2 α staining was also observed. However, this staining appeared less intense than the epidermal surface. Similarly, AP-2 α staining was detected in the conjunctiva at P1 but with a somewhat decreased intensity as compared to E12.5 (data not shown). Immunolocalization with the AP-2 β antibody showed reactivity in the keratinized layer of the eyelids, some of the mesenchymal tissue of the eyelid dermis, and the corneal epithelium (Fig. 1C).

Reopening of the murine eyelid is completed by post-natal day 12 (Teraishi and Yoshioka, 2001). Following eyelid opening, we examined the expression of AP-2 α and AP-2 β in the adult eyelid. Similar to P1, AP-2 β expression was mainly limited to the mesenchymal tissue, whereas AP-2 α expression was strongly detected in the more basal cell compartments of the eyelid epidermis and in the underling hair follicles (Fig. 1D).

In the adult cornea, AP-2 β protein was found to be expressed throughout all of the cells of the stratified layers of the corneal epithelium (Fig. 1F). In comparison, AP-2 α protein had a more restricted expression pattern, predominantly localizing to the nuclei of cells within the true basal cell compartment, as well as the nuclei of some cells within the stratum spinosum layer

(Fig. 1E). Overall, in contrast to the AP-2 α immunostaining, the AP-2 β staining did not appear to be nuclear.

Alterations in AP-2 α levels can affect expression and behavior of specific cellular genes

The more restricted expression pattern of the AP-2 α protein in the corneal epithelium and its predominant expression in the eyelid epithelium suggested that it may play a role in differentiation of the ocular surface tissues. To explore a role for AP-2 α in the corneal epithelium, the AP-2 α gene (human) was overexpressed in an ocular epithelial cell line, SIRC. SIRC cells are a spontaneously immortalized cell line derived from the rabbit cornea epithelium (Nieder Korn et al., 1990). These cells have lost some epithelial characteristics (Nieder Korn et al., 1990), and they express low to undetectable levels of AP-2 protein. However, like epithelial cells, they grow in adhesive colonies and express Pax6 protein (Sivak et al., 2000). Thus, the SIRC line was considered an optimal cell line for examining the effects of AP-2 α gene expression on cell behavior. In order to examine the effect of increased dosage levels of AP-2 α , SIRC cells were transfected with the AP-2 α gene and a number of stably-transformed lines were cloned. Some of the SIRC cells were also transfected with vector (pcDNA3) alone, and these were considered as the parent control cells. A number of the clones that were transfected with pcDNA3-AP-2 α vector induced levels of AP-2 α mRNA (Fig. 2) relative to the controls cells which translated into an increase in AP-2 α protein (see Western blot shown in Fig. 5). The increased expression of AP-2 α molecules was also able to functionally bind DNA as determined by supershift analysis (not shown).

The cells in many of these lines exhibited a clumping growth behavior very different from the smooth monolayer growth characteristic of the parent cell lines (Fig. 3). The severity of the clumping phenotype of the different clones also correlated with the levels of AP-2 α expression. For example, three strong expressing lines, P, L, F (Fig. 3), had severe phenotypes. The cell appearance of the AP-2 α transformed cell lines suggested that they had undergone a change in their capacity to adhere to or spread in the presence of serum attachment factors. To test this hypothesis, we assayed ECM adhesive properties of the different transformed lines using a Cytomatrix adhesion assay (Chemicon). Several of the clones stably transformed with wild-type AP-2 α had a significantly reduced ability to adhere to vitronectin and collagen type I in comparison to the parent cell line (Fig. 4), and this corresponded well with the altered cell phenotype. No difference was found for the other matrices tested including fibronectin, laminin, collagen type IV, and tenascin. Since the β 1 family integrins are the predominant collagen receptors present on epithelial cells, we examined whether the AP-2 α altered clones exhibited any change in β 1 gene expression. Lysates from control SIRC cells and the clones were tested with Western blot analyses using an antibody against β 1 integrin. These studies did not reveal any differences in integrin expression between the clones and control cells (data not shown).

The clustering phenotype observed in the SIRC clones, in combination with earlier studies which reported E-cadherin promoter binding of AP-2 α (Behrens et al., 1991), suggested the possibility of an alteration in expression of molecules involved in cell adhesion such as the cadherins. A screen of cadherin expression in the SIRC clones was performed using specific cadherin antibodies to both N-Cadherin (Fig. 5) and E-cadherin. To our surprise, we did not detect E-cadherin expression in the SIRC cell lysates, as was expected since corneal epithelial cells typically express E-cadherin (data not shown). Instead, we found that the SIRC cells constitutively expressed the 135–140 kDa N-cadherin protein (Fig. 5). These experiments further revealed that, in the transformed clones, N-cadherin expression was substantially upregulated (2.8 and 5.9 fold) in comparison to the parent cell line (controls) (Fig. 5). AP-2 α levels were also co-localized on the same blot to show a positive correlation between increased

AP-2 α protein levels and upregulated levels of N-cadherin. Thus, overexpression of AP-2 α in the SIRC cell line results in a corresponding upregulation of N-cadherin expression.

Cadherin expression in AP-2 α chimeric mice

As outlined earlier, our ability to investigate the role(s) of AP-2 genes (AP-2 α and AP-2 β) in differentiation of the ocular surface tissues using the current null mouse mutants is hindered either by earlier developmental defects or by lethality at birth, events which precede final differentiation of the eyelids and the cornea (Moser et al., 1997a; West-Mays et al., 1999). Chimeric mice, derived from populations of AP-2 α ^{-/-} and AP-2 α ^{+/+} cells, can be used to determine the ability of the knockout cells to populate and contribute various tissues in the developing embryo. In a previous study, we demonstrated using the AP-2 α chimeric mice that eye defects, similar to that observed in the null mice, could appear independent of craniofacial defects, confirming an important role for AP-2 α in eye development. In the current study, we have continued to use this model to determine whether the presence of the AP-2 α ^{-/-} cells in the ocular surface, as determined by lacZ expression or AP-2 α antibody staining, correlated with an altered expression of the cadherins. Immunohistochemistry with antibodies specific for either N-cadherin or E-cadherin were applied to sections of chimeric (E18) embryos. While immunostaining for N-cadherin in the ocular surface of the chimeras was similar to wild-type mice (data not shown), the pattern for E-cadherin was disrupted. E-cadherin staining was observed in all of the stratified layers covering the eyelids of chimeras but was strongest in the basal epithelial cell layer. In some chimeras, however, the E-cadherin staining in the eyelid was intermittent (Fig. 6A) and the locations of discontinuity strongly overlapped with regions of the epithelium that contained high populations of AP-2 α ^{-/-} cells, as determined by both LacZ staining (Fig. 6B) and AP-2 α antibody staining (Fig. 6C) on adjacent serial sections. The lack of E-cadherin staining and increased populations of AP-2 α ^{-/-} cells was also correlated with the abnormal, prenatal open-eyelid phenotype reported previously (Nottoli et al., 1998; West-Mays et al., 1999). The fact that very few AP-2 α ^{-/-} cells contributed to the developing corneal epithelium suggested that these cells could not be rescued by neighboring wild-type cells and that AP-2 α may have a cell autonomous role in corneal epithelial development. As a consequence of the few numbers of AP-2 α ^{-/-} cells in the chimera corneas, it was not possible to determine if cadherin would also be altered in this ocular surface tissue.

Discussion

Differentiation of stratified squamous epithelial tissues like the corneal epithelium and the epidermis of the skin involve multiple alterations in gene expression in order to enable the basal cells to leave the basement membrane and migrate to the surface where they are shed. Central to the changes required for terminal differentiation of the basal cells are a cessation of cell division and alterations in adhesive properties. The underlying cue(s) for terminal differentiation of these cells is not well understood.

AP-2 transcription factors have been implicated in regulating genes involved in epithelial differentiation, and defects in null mutant mice have revealed a requirement for AP-2 α in early development of the ocular epithelium (West-Mays et al., 1999; Hilger-Eversheim et al., 2000). In the current study, we have shown that the two AP-2 (α , β) proteins are differentially expressed in the developing ocular surface. AP-2 β protein was found to be broadly expressed throughout all of the stratified layers of the corneal epithelium, whereas AP-2 α expression was more restricted to the basal cell layers including the stratum basale and the stratum spinosum. AP-2 α protein expression was also highly expressed in the developing eyelid epidermis with particularly strong staining in the more basal cells of the epidermal surface. In contrast, AP-2 β expression was not detected in these tissues. Stable transfection of a corneal epithelial cell line with the AP-2 α gene resulted in an alteration in cell phenotype and an increase in

levels of a 135–140 kD cadherin protein identified as N-cadherin. Examination of the ocular surface of AP-2 α chimeric mice further revealed a positive correlation between the knockout cell location and regions of absent E-cadherin expression. Together, these findings suggest that AP-2 α participates in differentiation of the ocular surface through the regulation of cadherin gene expression.

The more restricted expression pattern of AP-2 α protein in the more basal cell compartments of the ocular surface epithelium as compared to AP-2 β suggests that it is positioned to contribute to the mechanisms which control differentiation. A number of genes involved in differentiation exhibit a differential expression pattern within the stratified layers of epithelial tissues, such as members of the integrins, keratins, and importantly, the cadherins (Schermer et al., 1989; Maytin et al., 1999). For example, E-cadherin expression in the epidermis is lost in the upper keratinized layers, and as a result, the decreased cell-cell adhesion is thought to contribute to terminal cell differentiation. In the cornea, the keratin 3 (K3) gene is expressed in the upper stratified layers, whereas its expression is repressed in the undifferentiated basal epithelial cell compartment. In a culture model for corneal epithelial cell differentiation, AP-2 was shown to act as a repressor of K3 expression (Chen et al., 1997). A down-regulation in AP-2 binding activity in the more suprabasal cells was found to coincide with differentiation and activation of K3. Paradoxically, the decrease in binding activity for AP-2 in the suprabasal cells was reported despite findings of a steady-state level of AP-2 mRNA throughout all layers of the cultures. Based on our *in vivo* findings that the AP-2 proteins are differentially expressed in the corneal epithelium, with AP-2 α expression restricted to the more basal cell layers, it is reasonable to propose that the repression of the K3 gene in the cultures was due to specific binding of AP-2 α .

Additional studies support a role for AP-2 α in regulating genes involved in differentiation. A subtractive hybridization screen of AP-2 α -/- mutant mice revealed premature expression of a target gene involved in the induction of terminal differentiation of embryonic fibroblasts, the Kruppel-box transcription factor, KLF-4 (Pfisterer et al., 2002). Similarly, our group has recently demonstrated that ectopic expression of AP-2 α in the developing lens resulted in repression of Membrane Intrinsic Protein (MIP) expression and inhibition in fiber cell differentiation (West-Mays et al., 2002). AP-2 has also been implicated in regulating genes involved in the cell cycle and promoting proliferation (Bosher et al., 1996; West-Mays et al., 1999; Pfisterer et al., 2002). Thus, the findings of the current study, including the localization of AP-2 α to the more undifferentiated cell populations of the ocular surface, support a growing body of evidence that AP-2 α may act in the maintenance of cells in the cell cycle and in preventing them from undergoing premature differentiation.

Overexpression of AP-2 α in SIRC cells resulted in a significant alteration in cell phenotype and gene expression profile. SIRC cells are generally fusiform in shape and form a confluent monolayer. In the stable clones, the cells clustered and did not plate well. A number of genes may have been altered in these clones and responsible for the observed phenotype. While Western blots indicated similar levels of β 1 family integrins, changes in integrin activation cannot be ruled out. We did, however, observe an alteration in the levels of as N-cadherin, a 135–140 kDa protein belonging to the family of cell adhesion molecules, the cadherins. N-cadherin is a Ca⁺⁺ dependent cell adhesion molecule originally identified in mouse and chicken nervous tissues (Hatta et al., 1985) and is classically described as being involved in homophilic cell-to-cell adhesion (Hatta et al., 1987). Thus, induced expression of N-cadherin in the clones may have been directly responsible for the clustering phenotype observed in the SIRC clones. Such an increase in cell-to-cell contact may have further resulted in less cell surface area available for adhesion to matrix, thereby resulting in reduced ability of the SIRC clones to adhere to ECM. Alternatively, since cadherins have also been shown to mediate cell-to-matrix adhesion through cross talk with integrin molecules (Hodivala and Watt, 1994:

Higgins et al., 1998), a change in the levels of N-cadherin may have altered the ability of the integrins expressed in the SIRC clones to interact with particular ECM molecules.

Decreased staining for E-cadherin was detected in the ocular surface tissue regions of AP-2 α chimeric mice that contained a high proportion of AP-2 α ^{-/-} cells. Regulation of E-cadherin promoter activity by AP-2 α has been shown previously in cultured keratinocytes (Behrens et al., 1991; Faraldo et al., 1997), and a positive correlation between localization patterns of AP-2 and E-cadherin expression have also been reported *in vivo* (Baldi et al., 2001). Recent findings by our group have further shown that ectopic expression of AP-2 α in the developing ocular lens resulted in expanded expression of E-cadherin protein in the lens transitional zone, the region in which epithelial cells terminally differentiate into fiber cells (West-Mays et al., 2002). Thus, E-cadherin is likely to be an important downstream target for AP-2 α during differentiation. We also found that overexpression of AP-2 α in a corneal epithelial cell line resulted in an upregulation of N-cadherin protein expression. Earlier characterization of the promoter region of the chicken N-cadherin gene had revealed several consensus AP-2 binding sequences which were required for promoter activity (Li et al., 1997). This suggests the possibility that AP-2 α may control N-cadherin expression through direct transactivation of the promoter, although this remains to be determined. Together, these findings suggest that AP-2 α may regulate the expression of multiple members of the cadherin family.

Corneal epithelial cells have been shown to express the classical cadherins, E-cadherin and P-cadherin (Takahashi et al., 1992; Mohan et al., 1995). Reports of N-cadherin expression in the cornea typically include that found in the neural crest cells which contribute to the corneal stroma and endothelium (Takeichi, 1988). Although transient N-cadherin expression has been reported in the corneal epithelium during embryonic development (Hatta et al., 1987), N-cadherin is not usually detected in the adult corneal epithelium. Thus, our observation of a low level of N-cadherin expression in the SIRC cells and no E-cadherin expression was unexpected and may suggest that this cell line does not express all corneal epithelial cell specific characteristics. In fact, SIRC cells have been known to lack other corneal epithelial specific genes (Nieder Korn et al., 1990). However, like other ocular epithelial cells, SIRC cells express Pax-6 protein (Sivak et al., 2000) and form adhesive colonies. The SIRC clones (transfected with AP-2 α) also formed circular clusters that closely resembled lentoid bodies, differentiated fiber cells derived from cultured lens epithelial cells, another ocular epithelial cell type. Thus, one possibility is that SIRC cells more closely resemble lens epithelial cells. Since AP-2 α acts in a combinatorial fashion with multiple transcription factors in governing epidermal gene expression (Sinha et al., 2000), the final outcome as to which of the cadherin subclasses is ultimately expressed in an ocular epithelial tissue is likely dependent on the milieu of additional factors expressed in each cell type. Further studies examining the factors that AP-2 interacts with and how this influences cadherin gene expression should help shed light on how the cadherins are differentially expressed in the developing and adult ocular epithelia.

In summary, we have shown that the AP-2 α and AP-2 β proteins are uniquely expressed in the differentiating ocular surface, with AP-2 α expression correlated with the more undifferentiated cell compartments. Altered expression of the AP-2 α gene also resulted in defects in differentiation of the corneal epithelium and stratified epithelium of the eyelids, and this was positively correlated with altered cadherin expression. Together, these findings suggest that AP-2 α acts as an important switch for regulating differentiation of the stratified epithelium of the ocular surface.

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Fig. 1. Immunolocalization of AP-2 α and AP-2 β in the developing ocular surface. (A) At E12.5, AP-2 α protein was expressed in the epithelial surface tissue adjacent to the eye region which will later form the eyelid epidermis (EY) and the conjunctiva (CO). A Rhodamine-conjugated secondary antibody was used to show specific staining of AP-2 α . Magnification: ca. 200 $\times$. (B) At P1, AP-2 α expression was observed in the eyelid epidermis with intense staining in the basally located cells of the epidermal surface. Fewer cells in the more suprabasal, stratum granulosum layer (SG) showed reactivity to AP-2 α , and no staining was observed in the superficial keratinized layers (K) of the developing eyelids. AP-2 α was also detected in the immature hair follicles just beneath the epidermal layer (HF). A Rhodamine-conjugated

secondary antibody was used to show specific staining of AP-2 α . Magnification: ca. 100 $\times$. **(C)** Immunolocalization with the AP-2 β antibody showed reactivity in the keratinized layer of the eyelids epidermis, some of the mesenchymal tissue of the eyelid dermis, and the corneal epithelium (*arrow*). A FITC-conjugated secondary antibody was used to show specific staining of AP-2 β . Magnification: ca. 100 $\times$. **(D)** AP-2 α expression in the adult eyelid was observed in the basal cells of the epidermal surface (*arrow*) and in the underlying hair follicles (*HF*). A Rhodamine-conjugated secondary antibody was used to show specific staining of AP-2 α . Magnification: ca. 100 $\times$. **(E, F)** In the adult cornea, AP-2 α (*E*) protein was detected in the more basally situated cells, whereas AP-2 β was expressed throughout all of the stratified layers (*F*). A Rhodamine-conjugated secondary antibody was used to show specific staining of AP-2 α , and a FITC-conjugated secondary antibody was used to show specific staining of AP-2 β . Magnification: ca. 200 $\times$.

Fig. 2.

Graphical representation of Northern blot data for AP-2 α mRNA expression in control SIRCs and SIRC clones. Control cells express nearly undetectable levels of AP-2 α mRNA relative to levels of GAPDH. Clones *P* and *L* express 9.1- and 8.4-fold induction of AP-2 α mRNA, respectively, over controls.

CON **P** **L** **F**

Fig. 3. Phenotype of control SIRC cells and SIRC cells stably transfected with the AP-2 α mini-gene. Control cells (*Con*) are fusiform in shape and plate as a monolayer. Cells transfected with the AP-2 mini-gene have an altered appearance (*P*, *L*, *F*) including a clustering/clumping phenotype. (Magnification: 20 $\times$).

Fig. 4. Adhesion of SIRC clones to substratum matrices. **(A)** The numbers of cells adhering to vitronectin for clone populations *P* and *L* were significantly less (*asterisk*) than control cells. **(B)** The numbers of cells adhering to collagen type I for clone populations *P* and *L* were significantly less (*asterisk*) than control cells.

Fig. 5. Immunoblot for N-cadherin and AP-2 α proteins expression in SIRC clones. Clones *D* and *L* which express significantly higher levels of AP-2 protein (49 kDa) than control cells also expressed significantly higher levels of N-cadherin (135 kDa). Control cells constitutively express low levels of N-cadherin. N1003A (lens epithelial) cell line was used as a positive control for both proteins examined.

Fig. 6. E-Cadherin expression in AP-2 α chimeric E18 embryonic mice. **(A)** E-cadherin staining was observed in all of the stratified layers covering the eyelid but was strongest in the basal epithelial cell layer. In some chimeras, however, the E-cadherin staining in the eyelids of E18 chimeric mice was intermittent (see *arrow* for absent staining). The E-cadherin antibody localization was revealed using an indirect Biotin/Avidin-Immunoperoxidase system. **(B)** An adjacent section to that shown in **A**, reveals high populations of AP-2 α ^{-/-} cells, as determined by LacZ (*blue*) staining. **(C)** An adjacent section stained for AP-2 α antibody also shows the location of the null cells (*arrowheads*). Magnification: ca. 100 $\times$.