Growth factors play important roles in regulating corneal epithelial cell proliferation/differentiation during wound healing. It is suggested that Pax6 involves corneal epithelial lineage-specific differentiation (Liu, J. J., Kao, W. W., and Wilson, S. E. (1999) Exp. Eye Res. 68, 295–301); however, the regulatory mechanism and function of Pax6 in growth factor-induced corneal epithelial responses is still unknown. In the present study, we found that the mitogenic effect of epidermal growth factor (EGF) in corneal epithelial cells required suppression of Pax6 activity through cellular mechanisms involving Erk-signaling pathway-mediated increase in CTCF expression. EGF-induced CCCTC binding factor (CTCF) activation subsequently inhibited Pax6 expression by interacting with a CTCF-specific region upstream of the pax6 P0 promoter. Suppression of EGF-induced Erk activation by specific inhibitor or by the dominant expression of a silent Erk mutant effectively abolished the effects of EGF stimulation on regulations of CTCF and pax6. Apparently, down-regulation of Pax6 expression induced by EGF is required for corneal epithelial proliferation, because overexpression of pax6 in these cells attenuated EGF-induced proliferation. In contrast, knockdown of mRNA expression with pax6- or CTCF-specific small interfering RNA in corneal epithelial cells significantly promoted or attenuated EGF-induced proliferation, respectively. Thus, our results revealed a new regulatory mechanism that involves cellular signaling events and pax6 transcription regulation in growth factor-mediated proliferation. In corneal epithelial cells, this suggests that inhibition of pax6 expression is a prerequisite for EGF to elicit controls of cell growth and fate.

Corneal epithelial cells on the surface layer of the cornea form the defense line as a barrier against noxious agents. Growth factor-mediated renewal of the corneal epithelium plays a functional role in maintaining the barrier function and corneal transparency (1, 2). The basal layer cells must proliferate at a fast rate to replace terminally differentiated cells in the more superficial layers and to maintain this protective function. Epidermal growth factor (EGF) is found to have stimulatory effects on corneal epithelial cell proliferation and migration (3). In wound healing models employing cultured corneal epithelial cells, the healing rate can be tremendously enhanced by an optimal dose of EGF present in the medium (4). EGF receptor-linked cell signaling in corneal epithelial cells includes pathways involving stimulation of phospholipase C, D, protein kinase A, phospholipase A2, phosphatidylinositol 3-kinase, and limbs of the mitogen-activated protein kinase cascade (5–12). Application of EGF in the serum starvation-synchronized corneal epithelial cells induces the formation of EGF receptor clusters in the cell membrane and increases cell proliferation by promoting cells entering $S$ and $G_{2}/M$ phases of the cell cycle (12, 13). The proliferative effect of EGF on corneal epithelial cell growth is a complex process including activation of the extracellular signal-regulated kinase limb of the mitogen-activated protein kinase cascade and stimulation of voltage-gated $K^{+}$ channels and bumetanide-sensitive Na-K-2Cl co-transporters (11, 13–16). However, the regulatory mechanism for the cell fade and mitogenic response to this cytokine still remains to be further investigated.

Pax6 plays a critical and evolutionarily conserved role in determining early stage cell differentiation in eye development in both vertebrates and invertebrates (17, 18). Homozygous mutation of eyeless, homologue of the pax6 gene, results in missing eye structures in Drosophila (19). Overexpression of pax6 can induce fully differentiated ectopic eyes and ectopic expressions of early eye development genes, such as Otx2, Rx, and Six3, as well as the endogenous pax6 (17, 20, 21). In addition, specific mutation of pax6 causes a small eye (Sey) defect in mice and ocular aniridia in humans (22–24). The Pax6 gene is expressed in essentially all ocular structures of vertebrates, including the cornea, iris, lens, and retina (25–28). Deficient expression of the pax6 gene causes death in mice shortly after birth. Although pax6 is down-regulated or fades away in most tissues and cell types following differentiation, it remains detectable in several mature cell types of the eye, including corneal, epithelial, lenticular, and retinal (29). In the developed cornea, PAX6 is a positive transcription factor essential for controlling the transcription of the cornea-specific differentiation marker keratin 12, suggesting that Pax6 may play an important role in early differentiation and maintaining a differentiation pattern in these cells (30).

The regulatory mechanisms of Pax6 gene expression are still largely unknown. It is apparently regulated by at least two enhancers. One has been identified as a 341-base pair enhancer located in the 5′ region 4.2 kb upstream of the pax6 gene (31). This enhancer is highly conserved in the Pax6 gene of mouse, humans, and puffer fish (Fugu) (31, 32). This enhancer is a dominant factor in the preplacodal phase of Pax6 expression.

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1 The abbreviations used are: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HCE, human corneal epithelial; RCE, rabbit corneal epithelial; PBS, phosphate-buffered saline; tet, tetracycline; CTCF, CCCTC binding factor; siRNA, small interfering RNA.

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and designated as the Pax6 ectoderm enhancer, whereas the existence of the other (enhancer 2) is implied. Recently, studies in our laboratory revealed that a repressor element is located at −1.2 kb upstream from the pax6 P0 promoter in mouse. The repressor element contains five repeat binding sites in the 80-bp region for CTFCF, a transcription factor binding to the CCCTC DNA sequence (33). Over-expression of the CTFCF gene in corneal cells induces a decrease of pax6 P0 activity, and a deletion mutant of the CTFCF binding sequence attenuated the decrease, indicating that CTFCF serves as a repressive protein in pax6 gene expression (33).

In this report, we present important results to demonstrate that the mitogenic effect of EGF in corneal epithelial cells requires suppression of PAX6 activity. The effect of EGF on the suppression of pax6 expression is through Erk-signaling pathway-mediated activation of CTFCF. EGF-induced activation of Erk cascades resulting in increases in CTFCF expression subsequently inhibited pax6 transcription. Overexpression of pax6 attenuated EGF-stimulated corneal epithelial proliferation, suggesting that inhibition of pax6 expression is a prerequisite for EGF to elicit control of cell growth and fate. In addition, our results revealed new regulatory mechanisms in corneal epithelial cells involving the EGF receptor-linked mitogen-activated protein kinase signaling pathway and transcription control of PAX6 function in growth factor-stimulated proliferation.

MATERIALS AND METHODS

Cell Culture and Gene Transfection—Human and rabbit corneal epithelial (HCE and RCE, respectively) cells were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium containing 10% fetal bovine serum and 5 µg/ml insulin (Sigma) in a 37 °C incubator gassed with 5% CO2. The growth factors that promote corneal epithelial proliferation. In studies in RCE cells, because EGF is one of the important growth factors that promote corneal epithelial proliferation. Western analysis in RCE cells. However, Pax6 was not expressed in RCE cells with 7.5% fetal bovine serum for 6 h (Fig. 1).

Northern Blot Experiments—Total RNAs were extracted with a gua- nidium solution (5 M guanidine hydrochloride, 50 mM Tris-HCl, pH 8, 0.5% N-lauroylsarcosine, 100 mM β-mercaptoethanol). Lysates were extracted three times with 50:50 phenolchloroform. Finally, RNAs were precipitated by centrifugation at 12,000 revolutions/min for 15 min after preincubation with ethanol at −80 °C. RNA (20 µg) for each sample was loaded in 1% agarose gel denatured with 2.2 M formaldehyde. The fractionated RNA was transferred onto nylon membrane. The membrane was subsequently hybridized with the corresponding α-32P-labeled DNA probe using a Random Primer labeling kit (New England Biolabs, Beverly, MA). Signals in the membrane were visualized by exposure to x-ray film at −80 °C overnight or longer.

Western Analysis Experiments—Western blot experiments were performed as described previously (36). In brief, 5 × 106 cells were harvested in 0.5 ml of lysis buffer (20 mM Tris, pH 7.5, 137 mM NaCl, 1.5 mM MgCl2, 2 mM EDTA, 10 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 10% glycerol, 1% Triton X-100, 1 mM Na- orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin). Cell lysates were prepared by centrifugation at 13,000 × g for 15 min. The cell lysates were denatured by adding equal volumes of 2× Laemmli buffer and by boiling for 5 min. Each sample with 20 µg of protein was electrophoresed in a 10% SDS-polyacrylamide gel and then transferred to polyvinylidene difluoride membrane. The membrane was incubated with rabbit anti-Pax6 or rabbit anti-Erk1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and then incubated with anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). Signals in the membrane were visualized using a horseradish peroxidase blot detection kit (Santa Cruz Biotechnology). All membranes were stripped following a standard stripping protocol. These membranes were washed twice with lysis buffer and then kinase buffer (20 mM HEPES, pH 7.6, 20 mM MgCl2, 25 mM β-glycerophosphate, 100 mM sodium orthovanadate, and 2 mM dithiothreitol). Immunoblots were resuspended in 100 µl of kinase buffer. Glutathione S-transferase/myelin basic protein (Santa Cruz Biotechnology) was used as a substrate for kinase activity assays. The kinase reaction was initiated by adding 2 µl of ATP mixture containing 20 µM ATP and 10 µCi of [γ-32P]ATP (Amersham Biosciences). The reaction was processed at room temperature for 5 min and terminated by adding 30 µl of 2× Laemmli buffer. Phosphorylation of myelin basic protein was detected by PAGE and visualized by autoradiography.

Analysis of β-Galactosidase (β-Gal) and Luciferase Activities—Pax6 P0 promoter and its mutant transfected cells were harvested for the Pax6 P0 promoter activity analysis in days 2 and 3 after transfection. The cells were washed twice with ice-cold PBS containing 5 µg/ml ice-cold lysis buffer containing 100 mM potassium phosphate buffer with 1 mM dithiothreitol, pH 7.8. Cells were disrupted by three cycles of freeze/thaw. Cell lysates were precleared by centrifugation at 13,000 × g for 5 min, and the supernatants were kept at −80 °C for later analysis of β-galactosidase activity. Galacton-star was used as a chemiluminescent substrate in a luminometer (Clontech, Palo Alto, CA) for determination of β-galactosidase activity. Chemiluminescent signals from products of Galacton-star catalyzed by β-galactosidase were determined by a luminometer. An internal control vector (pBRL-TK, 0.2 µg/transfection, Invitrogen) was introduced into the cells in parallel with pax6 P0 reporters for normalization of lacZ activity. The luciferase activity was measured using an assay kit supplied by Invitrogen. The Proliferation assay—The MTT cell proliferation assay is a colorimetric assay system that measures the reduction of a tetrazolium component (MTT) into an insoluble formazan product by the mitochondrion of viable cells. One hour prior to cell harvest, the culture medium was replaced with 1 ml of serum-free medium, and 100 µl of MTT solution (5 mg/ml in PBS) was added into each of the wells and incubated in a CO2 incubator for 1 h. The medium was replaced by 0.4 ml of acidic isopropyl alcohol (0.04 M HCl in absolute isopropyl alcohol) to solubilize the colored crystals. MTT was changed to a blue color by mitochondrial dehydrogenase. The samples were read using an enzyme-linked immunosorbent assay plate reader (Labsystems Multiskan MCC/340, Fisher Scientific) at a wavelength of 570 nm, with the background subtraction at 650 nm. The amount of color produced, normalized with the background, is directly proportional to the number of viable cells and is represented as the proliferation index.

RESULTS

Growth Factor-induced Increase in CTFCF Expression and Decrease in pax6 Expression—The effects of serum containing growth factors on expressions of CTFCF mRNA and PAX6 protein were tested by stimulating synchronized RCE and ML-1 cells with 7.5% fetal bovine serum for 6 h (Fig. 1A). Northern analysis revealed that the level of CTFCF mRNA was increased in response to serum stimulation in both RCE and ML-1 cells. There was a decrease in PAX6 protein expression detected by Western analysis in RCE cells. However, Pax6 was not expressed in ML-1 cells. To further investigate the regulatory mechanism, the effect of EGF on CTFCF and Pax6 activities was studied in RCE cells, because EGF is one of the important growth factors that promote corneal epithelial proliferation. The dose-response relationship and time course of EGF-stimulated increases in CTFCF mRNA expression were determined in RCE cells by Northern analysis (Fig. 1, B and C). Normalized density analyses obtained from three independent experiments were plotted to demonstrate the statistical significance, p < 0.05 (Fig. 1, B and C, lower panels). Expression of CTFCF apparently increased in response
to 10 ng/ml EGF stimulation at 3 h and reached the peak level at 12 h. On the other hand, control experiments detected a high expression level of basal pax6 protein in RCE cells using Western analysis. The effect of EGF on decrease in Pax6 expression demonstrated a dose-dependent pattern (Fig. 1D). Upon application of 10 ng/ml EGF, Pax6 expression was decreased following a time course and reached a very low level at 12 h (Fig. 1E). Statistical analysis showed the significant difference in EGF-induced changes of Pax6 expression (n = 3, p < 0.05) (Fig. 1, D and E, lower panels). These data suggest that there is a correlation between EGF-induced increase in CTCF expression and decrease in pax6 expression in RCE cells. This correlation is consistent with our previous report that CTCF can inhibit pax6 transcription by binding to a repressor element located upstream of the pax6 P0 promoter.

Effect of EGF on Pax6 Promoter Activity—In our previous studies, we have demonstrated that CTCF suppresses P0 activity through interaction with a repressor element (CTCF binding sites) located in the region −1.2 kb upstream from the pax6 P0 promoter (33). To verify whether EGF-induced decrease in Pax6 expression resulted from the increase in CTCF expression, the effect of EGF on Pax6 promoter activity was examined using the previously made P4.2 β-galactosidase reporter and an internal deletion mutant Pusahaan that lacks the binding sequences for CTCF (33). These reporter constructs were transiently introduced by electroporation into RCE cells and a stable RCE cell line that was previously transfected with tetracycline (tet)-inducible CTCF cDNA for overexpression of CTCF. To test Pax6 P0 promoter activity, P4.2 and Pusahaan mutant-transfected cells were induced with 2 μg/ml tetracycline. Tet-induced overexpression of CTCF markedly inhibited P4.2 promoter activity but did not affect Pusahaan mutant promoter activity (Fig. 2A). The effect of EGF on pax6 P0 promoter activity was also investigated in RCE cells that were transiently transfected with P4.2 and Pusahaan mutant reporters. EGF stimulation significantly inhibited P4.2 reporter activity but had no effect on Pusahaan mutant reporter activity, as the binding sequence for CTCF in the mutant reporter had been deleted (Fig. 2B). The inhibitory effect of EGF on the pax6 P0 promoter was rather specific, because there were no changes in β-galactosidase activity in RCE cells transfected with cytomegalovirus-β-gal control vector. The results indicate that EGF inhibits pax6 P0 promoter activity through increase in CTCF activity in RCE cells.

Effect of EGF-induced Erk Activation on CTCF and Pax6 Expression—Previous studies from our laboratory indicate that the Erk signaling pathway is one of the major EGF-induced signaling events responsible for RCE cell proliferation (11). Upon stimulation of RCE cells with 10 ng/ml EGF, Erk activity was transiently increased within 24 h (Fig. 3A). EGF-induced effects on CTCF
and Pax6 expressions were markedly affected by PD98059, which is a specific inhibitor for mitogen-activated protein kinase/extracellular signal-regulated kinase kinase, a mitogen-activated protein kinase kinase immediately upstream from Erk in the mitogen-activated protein kinase cascades (Fig. 3B). To further verify the effect of Erk on CTCF and Pax6 expressions, a constitutively active dominant negative Erk1 was established by double selections with zeocin and blasticidin. β-gal-Pax6 P0 reporter (P4.2-β-gal) and its internal deletion mutant (Pxba-β-gal) were transfected into tet-inducible RCE cells by electroporation. Promoter activities were detected and normalized by taking a ratio of measured β-galactosidase activity and internal control lacZ activity. The asterisk in the figure indicates the significant difference (n = 6, p < 0.01). ctr, control; LacI/Lac, ratio of β-galactosidase/luciferase activity.

Fig. 2. Effects of tet-inducible CTCF overexpression and EGF stimulation on pax6 P0 promoter readouts. A, effect of tet-inducible (Tet) overexpression of CTCF on pax6 P0 promoter activity. RCE cells were transfected with tet-inducible pcDNA4/To/A-CTCF containing full-length cDNA encoding CTCF. The stably transfected cells were established from a single colony by double selection procedures with zeocin and blasticidin. β-gal-Pax6 P0 reporter (P4.2-β-gal) and its internal deletion mutant (Pxba-β-gal) were transfected into tet-inducible CTCF RCE cells by electroporation. The asterisk in the figure indicates the significant difference (n = 6, p < 0.01). ctr, control; LacI/Lac, ratio of β-galactosidase/luciferase activity.

DISCUSSION

Corneal epithelial progenitor cells proliferate and differentiate to form the corneal epithelial layer by making spatial and temporal fate decisions. This renewal process maintains the healthy condition through a dynamic wound healing process. These processes are largely controlled by intercellular signal pathways through activation of growth factor receptors. Our data indicate that serum-containing growth factors, such as EGF, stimulate RCE cell proliferation in the absence of EGF and even much faster growing in EGF-induced cells in days 2 and 3.

Effect of Knocking Down CTCF mRNA on pax6 Expression and EGF-stimulated Proliferation.—To further verify the functional role of CTCF in mediating EGF-induced alteration of Pax6 transcription and RCE cell proliferation, CTCF activity in RCE cells was suppressed by knockdown of CTCF mRNA using CTCF-specific siRNA. As it has been shown in Fig. 1, EGF induced increases in CTCF expression and decreases in pax6 expression. However, EGF stimulation (20 ng/ml) failed to induce the increase in CTCF expression and decrease in pax6 expression in RCE cells that were transfected with CTCF-specific siRNA, (Fig. 5A). The effect of knocking down CTCF mRNA on CTCF and Pax6 expression were plotted as means with S.E. bars in Fig. 5, B and C, respectively. The significant difference was determined by analysis of variance (n = 3, p < 0.05). The effect of knocking down CTCF mRNA on EGF-induced RCE cell proliferation was also examined by MTT and presented as the proliferation index (Fig. 5D). Apparently, suppression of CTCF mRNA expression significantly inhibited RCE cell growth in both the absence and presence of EGF stimulation (n = 6, p < 0.05). Results from inhibition of CTCF mRNA expression experiments provide further evidence that EGF-induced RCE cell proliferation requires down-regulation of Pax6, and this action was mediated by CTCF.

Functional Role of Pax6 in EGF-stimulated RCE Cell Proliferation.—Function role of Pax6 in EGF-induced RCE cell proliferation was studied by overexpression of the pax6 gene and by knockdown of Pax6 mRNA. RCE cell proliferation was determined by MTT and presented as the proliferation index. A cDNA fragment encoding the full-length human pax6 gene was subcloned into pcDNA4 vector that contains a cytomegalovirus promoter. The full-length human pax6 cDNA was transfected into RCE cells by electroporation. Western analysis of human pax6 cDNA-transfected cells revealed a new and larger molecular mass band (47 kDa) representing human Pax6 protein, but this band was absent in vector-transfected control cells (Fig. 4A). The other lower molecular mass band (43 kDa) was found in both human Pax6 cDNA- and vector-transfected cells, representing the endogenous rabbit Pax6 protein. Stimulation of EGF (10 ng/ml) induced a fast proliferation in days 2 and 3 in control RCE cells. However, the proliferative effect of EGF was significantly attenuated by overexpression of pax6 in human pax6 cDNA-transfected cells (n = 6, p < 0.05), suggesting that Pax6 plays a functional role in the inhibition of EGF-induced cell proliferation (Fig. 4B). In contrast, transfected RCE cells with siRNA specific to the pax6 gene knocked down the endogenous pax6 mRNA (Fig. 4C). Interestingly, knockdown of Pax6 expression with siRNA significantly promoted RCE cell proliferation in the control cells and in EGF-stimulated RCE cells in days 2 and 3 (Fig. 4D). The effect of knocking down pax6 mRNA on cell proliferation was statistically significant (n = 6, p < 0.05). In addition, it resulted in the growing of RCE cells in the absence of EGF and even much faster growing in EGF-induced cells in days 2 and 3.

Corneal epithelial progenitor cells proliferate and differentiate to form the corneal epithelial layer by making spatial and temporal fate decisions. This renewal process maintains the healthy condition through a dynamic wound healing process. These processes are largely controlled by intercellular signal pathways through activation of growth factor receptors. Our data indicate that serum-containing growth factors, such as EGF, stimulate RCE cell growth by inhibiting CTCF expression and decreases in Pax6 expression. EGF-induced increase in CTCF expression and decrease in Pax6 expression were plotted as means with S.E. bars in Fig. 5, B and C, respectively. The significant difference was determined by analysis of variance (n = 3, p < 0.05). The effect of knocking down CTCF mRNA on EGF-induced RCE cell proliferation was also examined by MTT and presented as the proliferation index (Fig. 5D). Apparently, suppression of CTCF mRNA expression significantly inhibited RCE cell growth in both the absence and presence of EGF stimulation (n = 6, p < 0.05). Results from inhibition of CTCF mRNA expression experiments provide further evidence that EGF-induced RCE cell proliferation requires down-regulation of Pax6, and this action was mediated by CTCF.
development. However, PAX6 is not expressed in most mature tissue types, except in the eye and pancreatic tissues. Up to now, it is still not clear what is the functional role of PAX6 during corneal epithelial proliferation/differentiation.

In the present study, we aimed to determine the functional role of PAX6 in EGF-induced proliferation. Two parallel experiments were performed in RCE cells to manipulate Pax6 activities and to verify the effect of Pax6 on EGF-induced proliferation. First, Pax6 activity was altered by overexpression of the human pax6 gene and by knockdown of pax6 mRNA. Second, pax6 transcription was altered by tet-inducible overexpression of CTCF and by knockdown of CTCF mRNA. We found that, in RCE cells, up-regulation of pax6 expression by overexpression of the human pax6 gene significantly suppressed EGF-induced
FIG. 4. Effects of overexpression and knockdown of pax6 on EGF-induced
RCE cell proliferation. A, overexpression of human PAX6 in RCE cells. RCE cells were
transfected with pcDNA4-pax6 construct containing full-length cDNA encoding the
human PAX6 gene. Endogenous PAX6 protein (43 kDa) and transfected human PAX6
protein (47 kDa) were detected by Western analysis. β-actin proteins were detected as
loading controls. B, effect of overexpression of pax6 on EGF-induced cell proliferation.
EGF (10 ng/ml) was applied to human PAX6-transfected RCE cells, and cell prolif-
eration was determined on days 2 and 3 by MTT cell proliferation assay. C, knockdown
of pax6 mRNA using siRNA specific to PAX6. Expression levels of endogenous pax6
mRNA were detected by Northern analysis in control and pax6 siRNA-transfected RCE
cells. β-actin levels were detected as loading controls. D, effect of knocking down Pax6
mRNA on EGF-induced cell proliferation. EGF (10 ng/ml) was applied to Pax6 siRNA-
transfected RCE cells, and cell proliferation was determined on days 2 and 3 by MTT cell
proliferation assay. * indicates significant difference by comparison in the absence and
presence of EGF stimulation on days 2 and 3 (n = 6, p < 0.05), and ** indicates signifi-
cant difference by comparison of transfected and untransfected cells (n = 6, p < 0.05).
EGF-induced Down-regulation of Pax6

![Diagram of CTCF and Pax6](image)

FIG. 5. Effects of knocking down CTCF mRNA on Pax6 expression and EGF-induced RCE cell proliferation. A, effect of knocking down CTCF mRNA on Pax6 expression in the presence and absence of EGF stimulation. Expression levels of β-actin served as the loading controls. B, effect of knocking down CTCF mRNA on EGF-induced increase in CTCF expression. * represents the significant difference of EGF-induced changes (n = 3, p < 0.05). C, effect of knocking down CTCF mRNA on EGF-induced decrease in Pax6 expression. * represents the significant difference of EGF-induced changes (n = 3, p < 0.05). D, effects of knocking down CTCF mRNA on EGF-induced RCE cell proliferation. * indicates significant difference by comparison in the absence and presence of EGF stimulation on days 2 and 3 (n = 6, p < 0.05), and ** indicates significant difference by comparison of transfected and untransfected cells (n = 6, p < 0.05).

Protein kinase/extracellular signal-regulated kinase kinase (an immediate mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (an immediate mitogen-activated protein kinase kinase upstream from Erk) with PD98059 markedly blocked EGF-induced up-regulation of CTCF expression and down-regulation of Pax6 expression. Finally, overexpression of the tet-inducible dominant negative Erk in transfected RCE cells effectively inhibited EGF-induced up-regulation of CTCF expression and down-regulation of pax6 expression. These results provide sufficient evidence that the regulatory effect of EGF on CTCF and subsequently on pax6 expression is through the EGFR-linked Erk signaling pathway.

In summary, the present work, for the first time, demonstrates three important findings. First, EGF induces increases in CTCF expression in corneal epithelial cells through activation of the Erk signaling pathway. It is known that Erk nuclear translocation occurs after activation and regulates gene expressions, and it is possible that CTCF is one of those genes regulated by Erk. Second, EGF induces increases in CTCF expression to suppress pax6 expression by inhibition of pax6 transcription. The inhibitory effect of CTCF is accomplished by interaction with a specific DNA binding region in the pax6 P0 promoter. Finally, our data demonstrate that knockdown of pax6 expression promotes EGF-induced cell proliferation. In contrast, overexpression of pax6 expression attenuates the EGF effect on RCE cell proliferation, suggesting that Pax6 plays an important role in controlling growth factor-induced corneal epithelial cell growth. Apparently, EGF-induced RCE cell proliferation requires down-regulation of pax6 to prevent premature differentiation.

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