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Conditional deletion of Cited2 results in defective corneal epithelial morphogenesis and maintenance

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ABSTRACT

Cited2 is an important transcriptional cofactor involved in multiple organ development. Gene profile analysis has identified Cited2 as one of the transcription factors expressed at high levels in adult mouse cornea. To address the function of Cited2 in corneal morphogenesis, we deleted Cited2 in surface ectoderm derived ocular structures including cornea by crossing Cited2-floxed mice with Le-Cre transgenic mice. Cited2$^{flox/flox}$; Le-Cre^{+} eyes invariably displayed corneal opacity and developed spontaneous corneal neovascularization at older age. Fewer layers of corneal epithelial cells and the absence of cytokeratin 12 (K12) expression featured Cited2 deficient postnatal and adult eyes. Cited2 deficient cornea exhibited impaired healing in response to corneal epithelial debridement by manifesting abnormal histology, lack of K12 expression and corneal neovascularization. Moreover, mechanistic studies suggest that Cited2 may play a role in corneal morphogenesis in part through modulating the expression of Pax6 and Klf4. Collectively, these findings demonstrate a novel function of Cited2 in postnatal corneal morphogenesis and maintenance. Our study will help better understand the molecular mechanisms involved in corneal biology, and more importantly, it may provide a valuable animal model for testing therapeutics in the treatment of corneal disorders, especially blindness as a result of corneal epithelial cell deficiency.

Introduction

Localized at the most anterior aspect of the eye, the transparent cornea is not only a major refractive component of the vision system, but also functions as the primary protective barrier against environmental insults. Defects in its development, maturation, infection or trauma can result in visual impairment. Corneal opacification, neovascularization, fibrosis and improper wound healing as a result of disease or injury have been recognized as the second-leading cause of blindness worldwide (Whitcher et al., 2001). Therefore, preservation of the integrity and transparency of the cornea is an essential component for vision. To achieve this, it is important to understand the molecular mechanisms responsible for the maintenance and restoration of corneal integrity in response to physiological and pathological injury, which in turn will facilitate the development of therapeutic strategies fighting the blindness caused by corneal dysfunction.

The cornea is composed of three major cellular layers: an outer epithelium, a central stroma and an inner monolayered endothelium. In mouse, the eyes open around postnatal day 14 and cells in the one- to two-cell-layered epithelium proliferate and differentiate to form a four- to five-cell-layered corneal epithelium by 21 days after birth. The mature corneal epithelium contains five to eight cell layers and the maturation process is achieved by 6 to 8 weeks after birth (Hay, 1979; Zieske, 2004). The superbasal corneal epithelial cells slough off on a regular basis and are replenished by corneal epithelial stem cells (Thoft and Friend, 1983; Collinson et al., 2002). The program of proliferation and differentiation is under meticulous control in the adult mouse, which is essential for corneal homeostasis as it allows the most superficial corneal epithelial cells to be continuously replenished by oligopotent stem cells distributed throughout the ocular surface (Majo et al., 2008).

The differentiation mechanisms responsible for replenishing corneal epithelial cells are believed to be associated with changes in gene expression. SAGE (Serial Analysis of Gene Expression) identified

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Cited2 (CBP/p300-interacting transactivators with glutamic acid (E) and aspartic acid (D)-rich tail 2) as one of the transcriptional regulators with higher expression in adult mature cornea versus postnatal day 9 mouse cornea (Norman et al., 2004), suggesting the potential involvement of Cited2 in adult corneal maturation and maintenance. Cited2 is a member of a newly identified family of transcriptional modulators (Sun et al., 1998; Shioda et al., 1997; Bhattacharya et al., 1999). It is a nuclear protein that binds directly with high affinity to the first cysteine–histidine-rich (CH1) region of the transcriptional cofactors p300 and CBP. Cited2 physically interacts with several nuclear receptors and transcriptional factors, such as peroxisome proliferator-activated receptor (PPAR) (Tien et al., 2004), LIM domain-containing transcription factor Lhx2 (Glenn and Maurer, 1999), AP-2 transcription factors (Bamforth et al., 2001), SMAD2/3 (Chou et al., 2006), HNF4 α (Qu et al., 2007) and WT1 (Val et al., 2007). Cited2 also functions as a negative regulator of hypoxia inducible factor (HIF)-1 mediated signaling by competing with HIF-1α for binding to CBP/p300 (Bhattacharya et al., 1999). Cited2 is induced by many biological stimuli such as cytokines, serum and LPS in different cell types (Sun et al., 1998). Overexpression of Cited2 in Rat1 cells results in loss of cell contact inhibition, anchorage-independent growth and tumor formation in nude mice, suggesting that Cited2 is a transforming gene (Sun et al., 1998). These in vitro studies underscore the potential roles of Cited2 in various biological processes. Targeted deletion of Cited2 is embryonic lethal with embryos manifesting developmental defects in multiple organs (Bamforth et al., 2001; Bamforth et al., 2004; Barbera et al., 2002; Buaas et al., 2009; Chen et al., 2007; Qu et al., 2007; Val et al., 2007; Weninger et al., 2005; Withington et al., 2006; Xu et al., 2008; Yin et al., 2002). It is worth noting that Cited2 is essential for eye development since Cited2 deficient eyes display lens stalk formation, hyaloid hypercellularity and aberrant hyaloid vasculature during embryonic development and postnatal life (Chen et al., 2008).

In the present study, we have observed corneal opacity, thinning of corneal epithelium, loss of differentiation marker K12 expression in the corneal epithelium, impaired corneal epithelial wound healing and spontaneous corneal neovascularization in Cited2 deficient eyes. Therefore, we have identified Cited2 as a novel molecule involved in postnatal corneal epithelial cell differentiation and maintenance.

Materials and methods

Mouse lines

Both Cited2$^{flox/flox}$ (Preis et al., 2006) and Le-Cre transgenic (Ashery-Padan et al., 2000) mice lines were maintained on the C57BL/6 background. Cited2$^{flox/flox}$ mice were bred with Le-Cre transgenic mice to generate Cited2$^{flox/flox}$;Le-Cre^{+} and Cited2$^{flox/flox}$; Le-Cre^{-} mice.

Histology

Enucleated eye balls were fixed in 10% formalin, dehydrated, embedded in paraffin and processed with 7 μm transverse sectioning. Histology of the eyes was examined by light microscopy after hematoxylin and eosin (H&E) staining.

Immunohistochemistry

Heads from embryonic day 17.5 (E17.5) embryos were fixed in 4% paraformaldehyde, equilibrated in 30% sucrose, embedded in O.C.T. and processed with 10 μm cryosectioning. Enucleated eye balls from postnatal and adult mice were fixed in 10% formalin and processed for paraffin embedding. Paraffin-embedded sections were deparaffinized first before processed for immunohistochemistry. Cited2 immunostaining was performed using primary antibody against Cited2 (sc25375, Santa Cruz) and Alexa Fluor 488-conjugated anti-rabbit secondary antibody (A11008, Invitrogen). K12 immunostaining was performed using antibody against K12 (sc17101, Santa Cruz) in conjunction with ABC kit (PK-4005, Vector Laboratory) and antibody staining was developed with 3, 3′-diaminobenzidine (Sigma). α-smooth muscle actin (α-SMA) immunostaining was performed with antibodies against α-SMA (A5228, Sigma) and antibody staining was visualized with Alexa Fluor 594-conjugated anti-mouse secondary antibody (A11005, Invitrogen).

Real-time PCR

Corneas were dissected from enucleated eyes of mice at 6 weeks old. Total RNA was extracted using TRIzol (Invitrogen) and reverse transcribed using Superscript II reverse transcriptase. Real-time PCR was performed to measure the mRNA expression levels of Pax6 (Chen et al., 2008) and Klf4 using the following primers: Klf4 forward 5′-ccaccaggactacccctaca-3′: Klf4 reverse: 5′-ggggacttgtgactgcatct-3′.

Wound healing assay

Mice at 6 weeks of age were anesthetized before performing the corneal epithelial debridement. The central corneal epithelium (2 mm in diameter) was demarcated with a 2 mm trephine and subsequently removed using an AlgerbrushII (Alger Co.) tipped with a 0.5 mm burr under a dissecting stereomicroscope. To prevent ocular surface desiccation, eyes were kept moisturized with methylcellulose until recumbent. The mice were sacrificed 7 days after the wounding and the eyes were enucleated and fixed in 4% paraformaldehyde for further histological and immunohistochemical examination.

Luciferase assay

HCE (Human Corneal Epithelial), NMuMG (Normal Murine Mammary Gland Epithelial) and HEK293 (Human Embryonic Kidney) cells were seeded in 24-well plates for transfection of 0.15 ng pRLSV40, 40 ng of 1Kb human Klf4 promoter-containing firefly luciferase reporter, various amounts of Cited2 expression plasmid and control plasmid so that the total amount of DNA was 0. 4 μg/well. 20 ng of various truncated mouse Klf4 promoter-containing firefly luciferase reporter constructs (Chen et al., 2002) was also tested in a similar manner. Fugene6 transfection reagent (Roche) was used for the transfection. Firefly and *Renilla* luciferase activities were measured 24 h after transfection using Dual-luciferase assay reagents (Promega) on luminometer. Relative luciferase activity was calculated by dividing firefly luciferase activity by *Renilla* luciferase activity.

Results

Le-Cre mediated conditional deletion of Cited2, a gene highly expressed in adult cornea epithelial cells, results in corneal opacity

The expression of Cited2 in adult mouse cornea was first demonstrated by SAGE analysis (Norman et al., 2004). To verify the protein expression of Cited2 in adult cornea epithelial cells, immunostaining was performed and revealed Cited2 nuclear localization mainly in the majority of basal corneal epithelial cells and some of the supra-basal cells (Figs. 1a, c). To explore the function of Cited2 in corneal epithelial cells, conditional deletion of Cited2 in the surface ectoderm derived tissues including cornea was performed by crossing Le-Cre transgenic mice (Ashery-Padan et al., 2000) with Cited2$^{flox/flox}$ mice (Preis et al., 2006) since targeted deletion of Cited2 results in embryonic lethality. Le-Cre is expressed in the surface ectoderm from embryonic day (E) 9.5 and in surface ectoderm derived structures including the developing lens, cornea, conjunctiva and skin of the eye lids. As expected, Cited2 nuclear localization was not detected in