Transgenic Mice Expressing Dominant-negative Osmotic-response Element-binding Protein (OREBP) in Lens Exhibit Fiber Cell Elongation Defect Associated with Increased DNA Breaks*

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Osmotic-response element-binding protein (OREBP), also known as TonEBP or NFAT5, is thought to be responsible for the induction of osmolyte-accumulating genes when cells are under hypertonic stress. Recent studies suggest that OREBP also plays a role in water reabsorption in the kidney, T-cell proliferation, and embryonic development. We developed transgenic mice that express the dominant-negative OREBP (OREB-dn) specifically in the lens because our earlier studies showed that it is particularly sensitive to osmotic stress. The transgenic mice developed nuclear cataract soon after birth, suggesting defects in lens development. The developing transgenic lenses showed incomplete elongation of fiber cells and formation of vacuoles. This is accompanied by evidence of DNA strand breaks, activation of p53, and induction of checkpoint kinase, suggesting that the developing fiber cells lacking OREBP are in a similar physiological state as cells experiencing hypertonic stress. These results indicate that OREBP-mediated accumulation of osmolytes is essential during elongation of the lens fiber cells.

The osmotic-response element-binding protein (OREBP), also known as TonEBP or NFAT5, is a member of the NFAT transcription factor family characterized by the presence of the Rel homology DNA-binding domain (1, 2). Unlike other members in the NFAT family that are responsible for effective immune response, OREBP/TonEBP is primarily involved in the genetic program that is essential for cellular survival in response to hypertonic stress (3). Through binding to the osmotic-response element (ORE) or the toxicity-responsive enhancer (TonE), OREBP/TonEBP mediates the hypertonic induction of a set of osmoprotective genes including aldose reductase, betaine/g-aminobutyric acid transporter, and sodium-myo-inositol cotransporter, resulting in the cellular accumulation of sorbitol, betaine, and myo-inositol, respectively (4–6). It is well established that these small molecule organic osmolytes are able to replace excess intracellular electrolytes that are otherwise deleterious to normal cell functions (7). This adaptive mechanism is particularly important in the kidney medulla, where cells are constantly exposed to steep osmotic gradient due to the urine concentration mechanism. Besides, OREBP is also responsible for the hypertonic induction of HSP70 (8), a molecular chaperone that plays a vital role in cellular adaptation to osmotic stress (9).

There are growing evidences suggesting that OREBP is involved in diverse cellular responses in addition to osmoprotection. In Drosophila, OREBP orthologue MESR1 was identified as a modifier of RAS1 signaling involved in eye development (10). On the other hand, OREBP was also implicated in cancer cell migration and cancer metastasis (11). In mouse embryo, OREBP is expressed in most developing tissues including brain, eye, heart, kidney, colon, and muscle. Since these developing organs are not expected to experience hypertonic stress, the physiological role of OREBP in these tissues remains elusive (12). Nevertheless, OREBP homozygous knock-out mice manifested mid-embryonic lethality, indicating the important role of OREBP during development. A few OREBP null mutant mice that managed to survive through parturition displayed growth retardation and perinatal lethality associated with severe renal abnormalities (13). We have created transgenic (Tg) mice expressing a dominant-negative form of OREBP (OREB-dn) specifically in the collecting duct epithelial cells of the kidney and found that OREBP also plays an essential role in urine-concentrating mechanism by regulating the expression of aquaporin-2 and urea transporter genes (14). Furthermore, OREBP was also suggested to be vital for optimal T cell proliferation and function as Tg mice expressing OREBP-dn in the T cells (15) or thymus isolated from the OREBP heterozygous knock-out mice (16) exhibit a dramatic reduction in thymic cell proliferation and T cell-mediated antibody response. The apparent correlation between OREBP activity and cell proliferation revealed by the studies from the OREBP-dn transgenic and OREBP null mice suggests that an intact cellular osmotic-response pathway is central to cell proliferation and function in some tissues. However, the underlying mechanism remains unclear. More importantly, the physiological function

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† The abbreviations used are: ORE, osmotic-response element; OREBP, ORE-binding protein; OREBP-dn, dominant-negative OREBP; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling; Tg, transgenic; TonE, toxicity-responsive enhancer; P, postnatal day; E, embryonic day; p-Chk2, phosphorylated Chk2; TonEBP, toxicity-responsive enhancer-binding protein.

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OUREP Is Essential for Lens Development

of OUREP in developing tissues other than kidney and lymphoid remains unexplored. We have previously shown that developing mouse lens is sensitive to osmotic imbalance (17). Therefore we developed Tg mice that express OUREPdn specifically in the lens fiber cells to determine the role of OUREP in lens development. Our data demonstrate, for the first time in vivo, that inhibition of OUREP activity is associated with increase in DNA breaks and activation of DNA damage-response pathway, leading to defect in lens fiber cells elongation and the development of nuclear cataract. Our findings suggest that, during lens development, OUREP-mediated osmolyte accumulation is essential for fiber cell elongation and maintenance of the integrity of their DNA.

MATERIALS AND METHODS

Mice were housed under diurnal lighting condition and allowed free access to food and water. The protocol of this study was reviewed and approved by the Committee on the Use of Live Animals in Teaching and Research in The University of Hong Kong.

Generation of OUREPdn Transgenic Mice—To develop Tg mice, the 390-bp fragment of mouse a A-crystallin promoter (from −341 to +49) (17) was fused to the human OUREPdn to direct expression of the transgene, specifically in the mouse lens. The FLAG epitope was added to the amino terminus of OUREPdn to facilitate detection of transgene expression. The DNA fragment containing the OUREPdn transgene was microinjected into mouse oocytes. Tg mice were identified by PCR analysis using antibodies against p53, FLAG, A-crystallin (Stressgen, SPA-223), and β-actin (Sigma, A5441). Tg mice were sacrificed by cervical dislocation and dissected under the dissection microscope. Total RNA (15 μg/lane) extracted from the lens and other tissues of four-week-old mice using TRI reagent (Molecular Research Centre, Inc.) was separated by agarose-formaldehyde gel electrophoresis and transferred onto a Hybridon-N+ nylon membrane. Hybridization was carried out using “P-labeled OUREPdn and glyceraldehyde-3-phosphate dehydrogenase cDNA, respectively.

Expression of OUREP during Lens Development—There is no information regarding the temporal and spatial expression of OUREP in the developing lens, although immunostaining experiments showed that OUREP is present in the lens and the brain of the developing mouse embryo starting at embryonic day 10.5 (12). To investigate the expression pattern of OUREP during mouse lens development, we prepared paraffin sections of mouse lenses ranging from embryonic day 11.5 (E11.5) to postnatal day 21 (P21). Immunocytochemical analysis of these sections revealed that OUREP was expressed in the epithelial cells from E11.5 to E16.5 (Fig. 1, A–C) but decreased significantly at E18.5 (Fig. 1, D–G). In addition, OUREP expression was localized to the primary fiber cells at E13.5 (Fig. 1B), and a higher level of expression was found at E16.5 (Fig. 1C). At E16.5, OUREP immunoreactivity was also detected in the elongating secondary fiber cells (Fig. 1C). On the other hand, OUREP expression was reduced in fiber cells at E18.5 (Fig. 1D). Furthermore, in postnatal lens, OUREP expression was further reduced and was limited to the secondary fiber cells near the equatorial region (Fig. 1, E–G).

Generation of Transgenic Mice That Express OUREPdn Specifically in the Lens Fiber Cells—The highly specific temporal and spatial pattern of OUREP expression suggests that it may play a role in lens development. Since we and others have shown that mutant OUREPdn, lacking the transactivation domain, inhibits the function of endogenous OUREP (1, 14, 15), we examined the role of OUREP in the lens by generating Tg mice with the human OUREPdn cDNA (14) fused to the lens-specific A-crystallin promoter that has been shown to direct the expression of heterologous genes specifically in the lens fiber cells (17, 26). A FLAG epitope was inserted at the amino terminus of the OUREPdn to facilitate the detection of transgene expression (Fig. 2A). Tg mice were identified by PCR and confirmed by Southern blot hybridization (data not shown). Eleven lines of Tg mice were developed, and two lines were found to express detectable levels of the transgene (lines 4157 and 4165). Northern blot analysis showed that the level of transgene expression in line 4165 is ~2-fold higher than that in line 4157. In both lines, the transgene expression was only present in the lens but not in other tissues tested, including the brain, lung, liver, and heart (Fig. 2B). Because both lines of mice exhibited similar nuclear cataract in the adult and abnormal embryonic lens morphology, we chose to characterize in detail line 4165, which has a higher level of transgene expression.

Phenotypic Analysis of the Transgenic Mice—The Tg mice showed bilateral nuclear cataract 2 weeks after birth, the earliest time examined, suggesting developmental defect in their lenses. To determine the onset of such phenotype, we examined the expression of the transgene and the morphology of the lens...
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Expression of endogenous OREBP and OREBPdn transgene. Expression of endogenous OREBP in embryonic (A–D) and postnatal (E–G) lenses in wild-type mice, and expression of OREBPdn transgene (FLAG) in embryonic (H–K) and postnatal (L–N) lenses in transgenic mice as detected by immunocytochemistry is shown. Note the presence of vacuoles in the Tg lens (J), as detected by immunocytochemistry is shown. Note the presence of vacuoles in the Tg lens (J and K). Arrows indicate lens epithelial cells. Arrowheads indicate remnant nuclei. Sections were counterstained with hematoxylin. Scale bar, A–D and H–K, 50 μm; E–G and L–N, 100 μm.

FIG. 1. Expression of endogenous OREBP and OREBPdn transgene. Expression of endogenous OREBP in embryonic (A–D) and postnatal (E–G) lenses in wild-type mice, and expression of OREBPdn transgene (FLAG) in embryonic (H–K) and postnatal (L–N) lenses in transgenic mice as detected by immunocytochemistry is shown. Note the presence of vacuoles in the Tg lens (J and K). Arrows indicate lens epithelial cells. Arrowheads indicate remnant nuclei. Sections were counterstained with hematoxylin. Scale bar, A–D and H–K, 50 μm; E–G and L–N, 100 μm.

in the early stages of development. FLAG immunoreactivity was not detected in the epithelial cells throughout lens development (Fig. 1, H–N). Transgene expression was detected in the lens primary fiber cells starting at E13.5 (Fig. 1J). The transgene became highly expressed at E16.5. At this stage, FLAG immunoreactivity was detected in the primary fiber cells, where the majority of the signal was found in the nucleus (Fig. 1J), as well as in the elongating secondary fiber cells, where the signal was in both the cytoplasm and the nucleus. The newly differentiated secondary fiber cells did not express the transgene (Fig. 1J). Expression of the transgene was associated with cellular deformities. At E16.5, numerous vacuoles were observed in the lens (Fig. 1J), and both primary and secondary fiber cells showed retarded elongation leading to an unfilled space in the anterior lens surrounding the primary fiber cell core. By E18.5, the transgene expression was markedly reduced and was localized only to the secondary fiber cells. Similar to the lenses at E16.5, numerous vacuoles were present (Fig. 1K). The level of transgene expression was significantly reduced in postnatal lens (Fig. 1, L and M). At P7, the transgene was only moderately expressed at the secondary fiber cells and restricted to the older elongating fiber cells (Fig. 1L). However, no vacuole or fiber cell defect was observed in all postnatal mouse lens sections. Interestingly, unlike the wild-type P7 lens in which the primary fiber cells were completely devoid of nucleus (Fig. 1E), the lenses of the age-matched Tg mice showed the presence of nucleus remnants in the fiber cells (Fig. 1L), suggesting a delay in the denucleation process.

To determine the effect of OREBP inhibition on postnatal lens development, mouse lenses were examined by slit-lamp microscopy. All Tg mice demonstrated bilateral nuclear cataract starting at 2 weeks after birth and persisted for the rest of their lives (Fig. 3A). Flat angle illumination revealed indentations on the anterior lens surface of the Tg mice (Fig. 3B). To examine lens morphology in detail, lenses from 2-week-old mice were excised and observed directly under dissection microscope. In the wild-type lens, the secondary fiber cell endings were attached to the anterior pole to form Y-shaped suture branches oriented at 120° to one another and were not resolvable under dissecting microscope (Fig. 3, C and D) (21). However, sutures of the Tg lenses were clearly visible and abnormally expanded. In addition, opacity was observed at the endings of the suture branches (Fig. 3, C and D). The expanded suture and opacity disappeared at the age of 3 weeks, leaving a refractive anomaly that was revealed by the lateral view of the lens under dissection microscope (Fig. 3E). Since the suture is formed by the merger of fiber cell endings, the above observations suggest that OREBPdn expression leads to retarded fiber cell elongation, consistent with the observations in embryonic lens.

Since defects in fiber cell development often result in microphthalmia, we measured the eye mass of the Tg mice. As shown in Fig. 4A, OREBPdn expression did not affect the growth of the mice since the body weights were similar between the Tg mice and their wild-type littermates as measured from weeks 1 to 8. However, eyes from the Tg mice were smaller than those from their wild-type littermates starting from week 1, and such difference persisted at least through 8 weeks (Fig. 4B). In addition, when the weight of the lenses from postnatal mouse was determined, the lenses of the Tg mice were also found to be lighter than those of their wild-type littermates at all stages by ~15% (p < 0.05). Similar mass differences were displayed in adult mice (Fig. 4C). Such difference was not due to the hydration status of the lens because the water content of the lens (~67%) was similar in both Tg mice and wild-type littermates (Data not shown).

Induction of DNA Breaks and Activation of DNA Damage-response Pathway in Tg Lens—It has been shown that hypertonic stress induces DNA double-strand breaks (22) and decreases cell proliferation that is associated with cell cycle delay in the S and G2–M phase (23). Moreover, acute elevation of osmolality with NaCl addition increases the total amount of p53 protein as well as in p53 phosphorylated on Ser-15 (24). We therefore examined whether inhibition of the osmotic-response pathway by OREBPdn expression activates the p53 tumor sup-
pressor pathway. Substantial increase in p53 protein level in the lenses of the newborn Tg mice was revealed by Western blotting, whereas the expression of H9251-B crystalline, which can be induced by hypertonicity (25), was not affected (Fig. 5). Immunohistochemical analysis revealed that p53 overexpression began at E16.5 when Tg expression is maximal (Fig. 6, A and B) and localized to both the primary and the secondary fiber cells of the Tg mice, suggesting that p53 expression is associated with OREBPdn expression (compare Fig. 6B and Fig. 1J). Furthermore, phosphorylated checkpoint kinase 2 (p-Chk2), the upstream kinase for the activation of p53, was also abundantly expressed in the fiber cells of the Tg mice (Fig. 6, C and D). Since DNA damage is one of the major mechanisms leading to Chk2 and p53 activation, resulting in cell cycle arrest and apoptosis (26), we examined apoptosis in the lens of the Tg mice. Using the conventional TUNEL assay, we failed to detect apoptotic fiber cells from E13.5 to E18.5 (data not shown). Nevertheless, when a modified TUNEL assay that is more sensitive in detecting DNA strand breaks was used (18, 19), we observed a remarkable increase in the incorporation of dUTP into the 3'-OH ends of DNA in the fiber cells of the Tg mice, suggesting extensive DNA breaks in OREBPdn-expressing lens (Fig. 6, E and F). Taken together, these data suggest that OREBPdn expression results in DNA damage, leading to the activation of p53-response pathway.

**DISCUSSION**

The ocular lens provides a unique system to study cell growth and differentiation because it contains only two types of cells, a core of fiber cells under an anterior monolayer of proliferative cuboidal epithelial stem cells. Distinct from many other cell types, the lens fiber cells are post-mitotic and terminally differentiated, yet these cells are directed to undergo significant elongation to complete lens development. We showed that OREBF is expressed in the epithelial and primary fiber cells during early lens development (E13.5). At this stage, the primary fiber cells are differentiating and elongating to fill the cavity of the spherical lens vesicle. OREBF expression was also found to be associated with the elongating secondary fiber cells at a later stage of embryonic development (E16.5 and E18.5) but reduced dramatically in postnatal lens. These data suggest that OREBF may play a role in lens development.

The OREBPdn Tg mice showed nuclear cataract before weaning. This is likely the result of impaired lens development. At the embryonic stage, the lenses of the Tg mice show numerous vacuoles, disorganized fiber cells, and incomplete elongation of fiber cells. Vacuoles are a common feature in many cataract models. The cause of vacuole formation is not clear. It is most likely due to degenerating fiber cells. Interestingly, the vacuoles in the fiber cells disappeared in the adult lenses, probably as a result of cell compaction during lens maturation. However, the damage to the primary fiber cells probably leads to disorganization of the crystallin structure and nuclear cataract. Since OREBPdn was only expressed in the fiber cells that were terminally differentiated, our findings mainly define the role of OREBF in cell growth and elongation. Elongation of fiber cells is an essential step in lens development. It is initiated by intracellular potassium accumulation, leading to osmotic water influx and the increase in cell volume (27). The

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**Fig. 3. Phenotype of the Tg lens.** Photographs of lens (P14) from wild-type (WT) and transgenic mice viewed under slit-lamp microscopy (A) and flat angle illumination (B) are shown. C and D, lens (P14) under dissecting microscope observed by bright-field and dark-field illumination, respectively. Note the region of opacity at the endings of the suture branches. E, lateral view of the lens (P21) under dissecting microscope. **Arrows** indicate nuclear cataract. **Arrowheads** indicate expanded suture. **Open arrow** indicates region of refractive anomaly.
failure of the fiber cells to complete their elongation process provides a clue to the function of OREBP. Perhaps OREBP, activated by developmental program rather than hypertonic environment, mediates rapid accumulation of osmolytes to build up intracellular osmotic pressure to facilitate water influx. On the other hand, it has been suggested that during the induction of cell growth, the massive synthesis of macromolecules and the concomitant depletion of amino acids would effectively decrease intracellular osmotic pressure, imposing osmotic stress to cells analogous to that of exposure of cells to hypertonicity (28). Fiber cells differentiation, in particular, is marked by a dramatic accumulation of lens crystallin proteins (28, 29), suggesting that OREBP is required to orchestrate the increase in osmolyte accumulation not only to fill in the void created by amino acid depletion but also to increase osmotic pressure for cell elongation. Unfortunately, determining the intracellular osmotic pressure or the levels of various osmolytes in the elongating fiber cells in the embryonic lenses is technically difficult.

One indication that the elongating lens fiber cells from the OREBPdn Tg mouse are experiencing hypertonic stress is the observation that they have increased DNA strand breaks similar to those observed in cells under hypertonic stress. It has been shown that exposure of kidney cells to acute hypertonic stress (600 mosM) causes DNA double-strand breaks (22), and widespread DNA strand breaks were also detected in the kidney inner medulla in vivo (30). This is concomitant with the induction of p53 and cell cycle delay (24, 31, 32). In lens fiber cells of the OREBPdn Tg mice, DNA damage was accompanied by the activation of the DNA damage checkpoint pathway, as evidenced by the phosphorylation of Chk2. This is different from cells under hypertonic stress in which DNA damage response, as marked by chk phosphorylation, is compromised (30). In proliferating cells, the Chk2 protein plays an important role in the DNA damage response.
role in organizing the DNA damage response including stabilization of p53 and phosphorylation of Cdc25A and Cdc25C proteins, resulting in cell cycle arrest at G_{1}/S or G_{2}/M or apoptosis (33,34). However, the expression of p53 in terminally differentiated fiber cells and the absence of apoptosis in the Tg lens argue against the role of p53 in cell cycle control of these cells. It is likely that the activation of DNA damage-response pathway may also account for the lymphoid hypocellularity in the OREBP knock-out mice (16), as well as the development of hydropnephrosis in the transgenic mice that express OREBDn in the kidney collecting duct epithelial cells (14).

In the lens of the adult mice, p53 immunoreactivity was found in the epithelial cells of the central and pre-equatorial zones, as well as in the lens fiber cells at the bow region (35). However, the physiological function of p53 in these cells has not been determined. Interestingly, transgenic mice overexpressing wild-type p53 developed microphthalmia associated with fiber cell malformation and nuclear cataract (36). Likewise, our transgenic mice also overexpressed p53 and developed similar, albeit less severe, microphthalmia, fiber cell defects, and nuclear cataracts. The similarity between these two mouse models suggests that p53 induction may play a prominent role in the phenotype of our Tg mice. Apparently, the activation of p53 also delays the denucleation program of lens fiber cells, as suggested by the presence of nuclear remnant in the lenses of the Tg mice. However, the role of p53 leading to these abnormalities in the lenses of these Tg mice is not clear. In summary, our study demonstrates that OREBP is important for fiber cell growth and elongation and reveals a possible mechanism linking a defective osmotic-response pathway to DNA integrity and cell growth.

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