Disruption of Rest Leads to the Early Onset of Cataracts with the Aberrant Terminal Differentiation of Lens Fiber Cells

Hitomi Aoki¹, Hajime Ogino², Hiroyuki Tomita³, Akira Hara³, Takahiro Kunisada¹*

¹ Department of Tissue and Organ Development, Gifu University Graduate School of Medicine, Gifu, Japan, ² Department of Animal Bioscience, Nagahama Institute of Bio-Science and Technology, Nagahama, Japan, ³ Department of Tumor Pathology, Gifu University Graduate School of Medicine, Gifu, Japan

Abstract

REST (RE1-silencing transcription factor, also called Nrsf) is involved in the maintenance of the undifferentiated state of neuronal stem/progenitor cells in vitro by preventing precocious expression of neuronal genes. REST expression was then decreased in developing neurons to down-regulate neuronal genes which allow their maturation. However, the function of REST during neurogenesis in vivo remains to be elucidated because of the early embryonic lethal phenotype of conventional Rest knockout mice. In order to investigate the role of REST in ocular tissues, we generated and examined the mice evoking genetic ablation to Rest specifically to neural tissues including ocular tissue. We used a Sox1-Cre allele to excise the floxed Rest gene in the early neural tissues including the lens and retinal primordia. The resulting Rest conditional knockout (CKO) and control mice were used in comparative morphological, histological, and gene expression analyses.

Rest CKO mice had an abnormal lens morphology after birth. The proliferation of lens epithelial cells was likely to be slightly reduced, and vacuoles formed without a visible increase in apoptotic cells. Although the aberrant expression of late onset cataract marker proteins was not detected, the expression of Notch signaling-related genes including a previously identified REST-target gene was up-regulated around birth, and this was followed by the down-regulated expression of lens fiber regulator genes such as c-Maf and Prox1. Rest CKO induces a unique cataract phenotype just after birth. Augmented Notch signaling and the down-regulated expression of lens fiber regulator genes may be responsible for this phenotype. Our results highlight the significance of REST function in lens fiber formation, which is necessary for maintaining an intact lens structure.

Introduction

The transcriptional repressor RE1-silencing transcription factor, REST (also known as neuron-restrictive silencer factor NRSF), was initially discovered as a negative regulator of neuron-specific genes in non-neuronal cells [1,2]. REST is expressed throughout early development and
represses the expression of neuronal genes by transcriptionally silencing their promoters in conjunction with CoREST [3]. The target genes of REST include a number of genes encoding ion channels, neurotrophins, synaptic vesicle proteins, and neurotransmitter receptors [4–6].

Previous studies have shown that the expression of Rest is down-regulated as neural stem cells (NSCs) differentiate into mature neurons, and is completely silenced in mature adult neurons [7]. The repressor function of REST indicates that it plays a central role in inhibiting the precocious expression of neuronal genes in NSCs. Furthermore, its down-regulation upon the receipt of neuronal differentiation cues has been shown to permit the robust expression of neuronal genes for terminal differentiation [7].

A previous study reported that the targeted mutation of Rest in mice resulted in the derepression of a neuron-specific tubulin gene in a subset of non-neuronal tissues [8]. Although dispensable for embryonic neurogenesis in vivo, Rest was indicated to play a role in suppressing the expression of neuronal genes in cultured neuronal precursor cells and developing non-neuronal tissues [9,10]. Rest null mice survive to E9 without obvious morphological defects, by which time all three germ layers and the neural tube forms, clearly demonstrating that neuronal progenitors have the ability to develop in vivo in the absence of Rest. However, Rest null mice die by E11.5, prior to which the growth retardation caused by widespread apoptotic cell death starts at approximately E9.5 [8]. This early embryonic lethality has precluded further analyses of the potential role of Rest in the maintenance and differentiation of neural and non-neural cells in vivo.

In addition to its involvement in neurogenesis, recent studies have suggested that REST modulates glial lineage elaboration by coupling neurogenesis and gliogenesis [11,12], and the breakdown of these processes accompanies neurodegenerative disorders. Neural crest derived enteric nerve cell specific reduction of acetylcholinesterase was detected in the Rest CKO mice to induce the failure of the gut function by underdeveloped cholinergic transmission in the enteric nervous system [13]. The disruption of REST and its target gene interactions has been detected in patients with Alzheimer’s disease [14], microcephaly [15], epileptic seizures [16], Huntington’s Disease [17], Down’s syndrome [18,19]. In these neurodegenerative disorders, REST dysfunction has been suggested to cause the aberrant expression of various genes including neuronal genes.

Eye development is attained by close interactions between retinal and lens primordia [20]. Since the neural retina is part of the central nervous system and the lens is one of the sensory placode-derived tissues, their development may be under the control of the Rest gene. Thus, using our established Rest conditional knockout system in mice, we attempted to identify a possible ocular phenotype caused by the lack of REST expression. This approach revealed a novel function of Rest in lens development.

**Materials and Methods**

**Animals**

Mice were handled in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All animal experiments were approved by the Animal Research Committee of the Gifu University Graduate School of Medicine. Rest^2lox/2lox^ mice were generated from the Rest^2lox/+^ ES cell line as described previously [9,21]. Sox1-Cre mice [9,22] were bred with Rest^2lox/2lox^ mice, Rosa26R-EGFP mice [23], or Rosa26R-LacZ mice [24] to generate compound transgenic mice. The Rest^2lox/+^ ES cell line was generated from F1 (129SvJae x C57BL/6; V6.5 line) mouse ES cells. Sox1-Cre mice, Rosa26R-EGFP mice, and Rosa26R-LacZ mice had the C57BL/6 background. The results obtained for Rest CKO mice (Rest^2lox/2lox^; Sox1-Cre+) were compared with those for Rest^2lox/2lox^; Sox1-Cre- mice and Rest^+/+^; Sox1-Cre+
mice as a control. Sox1-Cre+; Rosa26R-EYFP embryos were dissected at E9.5, EYFP signals were examined under a fluorescent stereomicroscope (SZX16, Olympus), and digital images were captured with an Olympus DP70.

**LacZ staining**

Sox1-Cre+; Rosa26R-LacZ embryos were dissected at E10.5. The methods used for LacZ staining have been described in detail previously [25,26]. Briefly, embryos were fixed for 10 min in 2% paraformaldehyde supplemented with 0.2% glutaraldehyde and 0.02% Tween-20. After three washes in PBS, embryos were stained overnight at 37°C in 10 mM phosphate buffer (pH 7.2) containing 1.0 mM MgCl$_2$, 3.1 mM K$_4$[Fe(CN)$_6$], 3.1 mM K$_3$[Fe(CN)$_6$], and 2 mg/mL X-Gal. The staining reaction was stopped by washing in PBS. Specimens were postfixed overnight with phosphate buffer containing 4% formaldehyde (pH 7.2). Sox1-Cre+; Rosa26R-LacZ eyes from P0 pups were dissected and embedded in OCT compound and used for frozen sections. Frozen sections were stained using the same protocol as that for embryos.

**Histology and immunohistochemistry**

The eyes were enucleated and fixed by immersion overnight in phosphate buffer containing 4% formaldehyde (pH 7.2). The methods used for histological analyses have been described previously [9]. Briefly, specimens were dehydrated with ethanol, soaked in xylene, and embedded in paraffin. Serial sections were prepared at a thickness of 3 μm using a Leica RM2125RT microtome (Leica Microsystems Inc., Bannockburn, IL) and stained with hematoxylin and eosin (HE). We used at least four eyes from two mice for each of the indicated genotypes and time points shown in Table 1. At least five and three sections for HE staining and immunohistochemistry, respectively, were prepared from one eye at appropriate intervals.

A HISTOFINE Kit (Nichirei Bio Science, Japan) or VECTASTAIN ABC System (Vector laboratories, CA, USA) was used for immunohistochemistry according to the manufacturer’s protocols with 3,3′-diaminobenzidine (DAB). Specimens were examined under an Olympus BX-51 microscope (Olympus, Japan). Images were captured with an Olympus DP70 digital camera.

The primary antibodies used in this study were as follows: anti-mouse Ki67 (1:500; TEK3, Dako-Cytomation, Glostrup, Denmark), anti-Prox1 (1:500; Millipore, MA, USA), anti-gamma Crystallin (1:1000; Bioss, MA, USA), anti-c-Maf (1:500; Novus, CO, USA), and anti-smooth muscle actin (1:500; SMA, Dako).

Histological evaluations were performed with the support of two experienced pathologists (H.T. and A.H.) who were blinded to the experimental data.

**In situ terminal dUTP-biotin nick end labeling of DNA fragments (TUNEL) method**

TUNEL staining was performed to detect apoptotic cells as described previously [25,26]. After being incubated in 20 μg/ml proteinase K (Sigma), the serial sections used for HE staining were immersed in TDT buffer (30 mM Trizma base, pH 7.2, 140 mM sodium cacodylate, and 1 mM cobalt chloride). TDT and biotinylated dUTP (both from Roche) were diluted with TDT buffer at concentrations of 0.15 e.u./ml and 0.8 nmol/ml, respectively. The solution was placed on the sections and then incubated at 37°C for 60 min. The sections were covered with streptavidin peroxidase (DAKO, Carpinteria, CA, USA) and stained with DAB as a substrate for peroxidase. Counterstaining was performed using Mayer’s hematoxylin.
Gene expression analysis

Whole lenses were dissected from enucleated eyes of each embryo or mouse at the indicated embryonic or postnatal days and used for total RNA purification. 6, 15, 5, 4, 3, and 6 mice of each respective genotype were used to prepare RNAs at E13.5, E15.5, E17.5, P1, P7 and 10W, respectively. Total RNA was prepared using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg of total RNA using the SuperScript First-Strand Synthesis System (TAKARA BIO, Japan) with oligo dT primers. Real time PCR was performed using gene-specific primers and SYBR Premix Ex Taq (TAKARA BIO) with Thermal Cycler Dice Real Time System (TAKARA BIO). Real-time quantitative PCR was performed using the relative standard curve method to quantify the target gene expression. For normalization, β-actin was used. The primer sequences used in qRT-PCR analyses were obtained from the PrimerBank (http://pga.mgh.harvard.edu/primerbank/).

Statistical analysis

Statistical differences were assessed using the Student's t-test. P values of <0.05 or <0.01 were considered significant. Error bars in graphs denote s.d.

Results

Conditional ablation of Rest by Sox-Cre results in a postnatal cataract-like phenotype

A previous study revealed that mice lacking the Rest gene died during early embryonic development [8]. In order to examine the effects of a Rest deletion in the central nervous system in vivo, we established mice containing floxed Rest alleles and Sox1-Cre alleles (Rest<sup>lox/lox</sup>; Sox1-Cre+, named Rest CKO) [9,21]. Sox1 is a transcription factor gene whose expression occurs around E7.5 to E8.5 in the neural tube [22] and subsequently persists in the developing neural retina and retinal pigment epithelium [27]. Sox1 expression also starts in the

| genotype | Rest CKO | control |
|----------|----------|---------|
| stage    | abnormal (No) | normal (No) | incidence (%) | abnormal (No) | normal (No) | incidence (%) |
| No. of pups at E13.5 | 0 | 7 | 0 | 0 | 11 | 0 |
| No. of pups at E14.5 | 0 | 8 | 0 | 0 | 11 | 0 |
| No. of pups at E15.5 | 0 | 9 | 0 | 0 | 14 | 0 |
| No. of pups at E16.5 | 0 | 8 | 0 | 0 | 11 | 0 |
| No. of pups at E17.5 | 0 | 7 | 0 | 0 | 5 | 0 |
| No. of pups at P0 | 17 | 6 | 73.91 | 0 | 39 | 0 |
| No. of pups at P1 | 12 | 3 | 80 | 0 | 8 | 0 |
| No. of pups at P3 | 11 | 1 | 91.67 | 0 | 13 | 0 |
| No. of pups at P4 | 9 | 0 | 100 | 0 | 6 | 0 |
| No. of pups at P5 | 6 | 0 | 100 | 0 | 7 | 0 |
| No. of pups at P7 | 3 | 0 | 100 | 0 | 3 | 0 |
| No. of pups at P10 | 4 | 0 | 100 | 0 | 2 | 0 |
| No. of pups at 2W | 3 | 0 | 100 | 0 | 3 | 0 |
| No. of pups at 4W | 4 | 0 | 100 | 0 | 2 | 0 |
| No. of pups at 8W | 21 | 0 | 100 | 0 | 23 | 0 |

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invaginating lens placode by E10.5 [23,28]. Consistent with these findings, the expression of Sox1-Cre-induced YFP started at approximately E9.5 in ocular tissue (Fig 1A–1C) and the Sox1-Cre-induced expression of LacZ was detected at E10.5 in the lens placode (Fig 1D and 1E). Histological analyses also showed that the majority of lens epithelial cells as well as neural retinal and corneal epithelial cells were LacZ-positive at P0, indicating the expression of Cre recombinase driven by the Sox1 promoter in the lens epithelium (Fig 1F–1H).

We found that Rest CKO mice were born without an apparent abnormal morphology as previously reported [9]. However, 8 weeks after birth, mice developed severe lens opacity, which blocks or changes the entry of light, affecting vision and a cataract is characterized by opacities (Fig 1I, 1J, 1L and 1M). Furthermore, these lenses were so fragile that they easily broke down after being removed from the eye (Fig 1K–1N).

Vacuole formation and reduced proliferation of lens epithelial cells in the Rest CKO lens

In order to characterize the Rest CKO phenotype at the histological level, we performed a HE staining analysis of eye tissues dissected at embryonic days (E) 13.5, E14.5, E15.5, E16.5, and E17.5, and postnatal days (P) 0, P1, P3, P4, P5, P7, P10, P14, 4 weeks (W), and 8W (Fig 2). The eyes of Rest CKO mice started to exhibit an abnormal lens morphology after birth (lens from a Rest CKO mouse in Fig 2P–2T and 2Z–2AD and that of a control in Fig 2K–2O and 2U–2Y), but not during embryogenesis (lens from a Rest CKO mouse in Fig 2F–2J and that of a control in Fig 2A–2E). The number and incidence of the lens phenotype were summarized in Table 1. Dysplasia appeared as the formation of a vacuole (arrows in Fig 2P) or gaps (arrowheads in Fig 2Q) in the peripheral part of the lens fiber. The size and number of these vacuoles or gaps then increased, while the central part of the lens fiber mass began to show an irregular alignment, which was revealed as deformed layer structures (lens from a Rest CKO mouse in Figs 2P and 3H and that of a control in Figs 2K and 3D).

On the other hand, other ocular tissues, the retina, cornea, and uvea, including the choroid, iris, and ciliary body, did not show any morphological abnormalities in their major structural components (eye from a Rest CKO mouse in Fig 3E–3G and that of a control in Fig 3A–3C). The lenses of all Rest CKO mice started to show amorphous and disorganized lens fibers by P7 (lens from a Rest CKO mouse in Fig 3P and that of a control in Fig 3L) and large vacuoles 8 weeks after birth (lens from a Rest CKO mouse in Fig 3X and that of a control in Fig 3T), without any obvious histological abnormalities in other ocular tissues (eye from a Rest CKO mouse in Fig 3M–3O and 3U–3W and that of a control in Fig 3I–3K and 3Q–3S).

Rest CKO is likely to interferes with the proliferation of lens epithelial cells

In order to investigate the mechanisms underlying vacuole or gap formation, the proliferation of lens epithelial cells was examined in the E15.5, P4, P7, and 10W lenses of CKO mice. A slight decrease in the number of proliferating Ki67-positive cells was observed in the lens epithelial layer at each time point (Fig 4A–4E), however, it’s not significant. This suggest that it might be hard to explain only in such small decrease in lens epithelial number would result in the rather major disruption of lens structure. Then, we also performed a TUNEL assay to detect apoptotic changes in the E15.5, P4, P7, and 10W lenses of CKO mice. However, no detectable increase in the number of apoptotic cells was observed in the lens epithelial layer, indicating that apoptosis did not play a major role in the deformed phenotype (Fig 4F–4J).
Expression of lens protein markers in Rest CKO mice

We examined the expression patterns of commonly used lens protein markers such as Cryg, Prox1, and c-Maf by immunostaining at various ages (Fig 5 and S1 Fig). The expression of Cryg was constantly abundant in lenses from Rest CKO and control mice from E13.5 to 8 weeks after birth (Fig 5B and S1B Fig), while that of Prox1 and c-Maf was slightly lower in Rest CKO mice than in control mice (I, J). Lenses dissected from the eyeballs of control mice maintained their shape and clarity (K). An eyeball (right in N) dissected from a 8-week-old (G). Sox1 vesicles. (F-H) LacZ staining of E9.5 (A-C). LacZ staining of the lens epithelium (H) as well as the corneal epithelium (arrow) and neural retina (arrowhead) of Sox1-Cre+; Rosa26R-LacZ mice at P0 (G). Sox1-Cre+; Rosa26R-LacZ control section was shown in F. (I-N) Exterior view of the eye and lens. (I, J, L, M) Exterior view of the eye. Lens opacity was observed in 8-week-old Rest CKO mice (L, M), but not in control mice (I, J). Lenses dissected from the eyeballs of control mice maintained their shape and clarity (K). An eyeball (right in N) dissected from a Rest CKO mouse and a lens after being dissected from another eyeball (Left in N) were also shown (N). Lenses dissected from Rest CKO eyes readily collapsed after their removal from the eyes (left in N).

Expression profiles of REST target genes in the developing lens

In order to elucidate the molecular mechanisms responsible for abnormal lens development in Rest CKO mice, we first assessed the expression of Rest mRNA from dissected developing lenses. The primer pair was set inside of the floxed exon 4 of Rest gene. As shown in Fig 6A, Rest mRNA levels were significantly reduced in E13.5 to E17.5 Rest CKO lenses and P1, P7 Rest CKO lenses. Rest mRNA detected in CKO mice might be originated from the contaminated non-lens tissues. When the other primer pairs [31] was used, still Rest mRNA was detected from the control, Rest mRNA levels were significantly reduced in E15.5 to 10 week Rest CKO (Fig 6C). Cre mRNA was detected as early as E13.5 and a concomitant reduction in Cre mRNA expression levels of at least 10-fold was observed (Fig 6B). No significant difference was observed in the expression of Sox1 between control and Rest CKO lenses (Fig 6E). Therefore, the phenotypic change observed in Rest CKO mice (Fig 1I–1N) appeared far later than the start of reductions in Rest. The expression of β-actin, which was determined as a quantitative control, remained constant in each developmental stage (S2A Fig). As expected, the expression of some neuronal genes known as targets of REST, such as Syt4, Tubb3, Calb1, Bdnf, and Stmn2 was significantly up-regulated after birth in the Rest CKO lens (Fig 6F–6J). The expression of REST has previously been investigated in the lens [31,32]. They demonstrated that the expression of REST and a splice isoform of REST/NRSE called REST4 lacking a transcription repression domain predominated during lens fiber differentiation. After investigating Rest4 expression using the primer pair specifically detect Rest4 transcript [31], we found that the expression of Rest4 was significantly up-regulated in Rest CKO lens from E15.5 to 10 week (Fig 6D). This suggests that the ablation of the floxed Rest DNA induced by Sox1-Cre affect the alternative splicing pattern of Rest mRNA to preferentially generate Rest4 transcript.

Genome-wide mapping of in vivo protein-DNA interactions previously revealed that Hes1 is an immediate target of the REST protein [33]. Genome-wide identification of novel REST binding sites by ChIP-PET technology also revealed that Hes1 has a REST binding site and is
Fig 2. The sequential histology of the lens at the indicated time points in Rest CKO mice and their control littermates. Lenses at E13.5 (A, F), E14.5 (B, G), E15.5 (C, H), E16.5 (D, I), E17.5 (E, J), P0 (K, P), P1 (L, Q), P3 (M, R), P4 (N, S), P5 (O, T), P7 (U, Z), P10 (V, AA), P14 (W, AB), 4W (X, AC), and 8W (Y, AD) in Rest CKO mice (F-J, P-T, and Z-AD) and their control littermates (A-E, K-O, and U-Y) were shown. Lenses from Rest CKO adult mice clearly showed an abnormal morphology in the lens cortex and/or bow region, where newly formed secondary lens fibers are present, by HE staining. Arrows and arrowheads indicate the formation of vacuoles and gaps in lens fibers, respectively. Scale bars represent 50 μm.

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In vivo genetic ablation of Rest leads to abnormal lens differentiation. Ocular tissues at P0 (A-H), P7 (I-P), and 8W (Q-X) in Rest CKO mice (E-H, M-P, U-X) and their control littermates (A-D, I-L, Q-T). The retina (A, E, I, M, Q, U), angle (B, F, J, N, R, V), cornea (C, G, K, O, S, W), and lens (D, H, L, P, T, X) were shown. The scale bar represents 50 μm.

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Fig 4. Cell proliferation, apoptosis, and cataract marker protein expression in Rest–deficient lenses in vivo. (A–E) An immunohistochemical analysis of Ki67-positive proliferating cells. No significant differences were observed in the distribution of Ki67-positive cells in the surface layer of the lens capsule, regardless of their genotypes (A–D). Although not statistically significant, the number of Ki67-positive cells/length in the surface layer of the lens capsule of Rest CKO mice was slightly lower than that of their control littermates (E). (F–J) A histological analysis of TUNEL-positive apoptotic cells. Almost no TUNEL-positive cells were detected in the surface layer of the lens capsule, regardless of their genotypes (F–I). No significant difference was observed in the number of TUNEL-positive cells between Rest CKO mice and their control littermates (J). (K–N) Immunohistochemical staining for the proteins known to be associated with age-related cataracts in humans. Virtually no SMA-positive cells were detected in the surface layer of the lens capsule, regardless of their genotypes. The scale bar represents 50 μm.

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Fig 5. Immunohistochemical detection of lens marker proteins in lenses from a Rest−deficient mouse in vivo.
HE staining (A) and immunohistochemical staining with antibodies against Cryg (B), Prox1 (C), and c-Maf (D) at E15.5.
one of the potential target genes (Dataset S4 in Johnson et al., 2008)[34]. As shown in Fig 6K, the expression of Hes1 was greater at birth in Rest CKO than in the control, which was consistent with the transcription repressor function of REST for the Hes1 gene [33].

Hes1 is an important key factor involved in Notch signaling. Therefore, we examined the expression of the genes constituting the Notch signaling cascade (Fig 6L–6N and S2B–S2D Fig). Among them, the expression of Notch1 was significantly up-regulated in the P1 lens of mice, while that of Notch2 and Notch4 was significantly up-regulated in P1, P7, and adult lenses (Fig 6L–6N). The expression of Notch ligands such as Jagged2 and Dll1 was maintained at low levels in the control, whereas that of Jagged2 was significantly and gradually up-regulated with age in the Rest CKO lens after birth and that of Dll1 was also significantly up-regulated in the Rest CKO lens after P1 (S2B–S2D Fig). These results suggest that the enhanced expression of Hes1 by the Rest CKO status in the lens activates Notch signaling through an increase in the Notch receptor and their ligands. The involvement of Notch signaling has been directly tested in the developing lens and Notch signaling has been shown to control the timing of primary fiber cell differentiation and is essential for secondary fiber cell differentiation [35]. Gain- or loss-of-function mutations in Notch signaling lead to the formation of a dysgenic lens, and profound postnatal degeneration has been reported in the lens of an induced Notch loss-of-function mutant [36,37].

Previous findings have also suggested that Cyclin D1 and Cyclin D2 as well as the p27 (Kip1) cyclin-dependent kinase (CDK) inhibitor act downstream of Notch signaling, thereby defining multiple critical functions for this pathway during lens development [35]. Therefore, we assessed the expression of CDK inhibitor genes such as p21 and p27. The expression of both of these genes was maintained at low levels in the control (S2A and S2B Fig), whereas that of p27 was significantly and gradually up-regulated with age in the Rest CKO lens after birth, similar to Jagged2 (S3B Fig), and that of p21 was also significantly up-regulated in the Rest CKO lens at all time points, except for E17.5 (S3A Fig). On the other hand, the expression of p57 and CyclinD1 tended to be gradually down-regulated in both control and Rest CKO lens (S3C and S3D Fig). We herein demonstrated that augmented Notch signaling under the influence of the Rest CKO status was sufficient to interfere with normal lens fiber development after birth.

Expression profiles of genes involved in lens fiber differentiation

We examined the expression of genes involved in lens development according to the classification by Ogino et al. (2012)[37]. The expression level of lens-specification gene, Pax6 was lower before birth and higher after birth in Rest CKO lens than in the control lens (S2E Fig). The expression level of Sox2 was lower at E17.5 and higher at P1 in Rest CKO lens than in the control lens (S2F Fig). Another lens lineage-specification gene, Six3 was also down-regulated in most of the developmental stages of the Rest CKO lens (S2G Fig). These fluctuations in each lens-specification gene by Rest CKO were very small and the lens-specification process was not likely to be affected by the conditional ablation of Rest, at least in the embryo (Fig 2).

The expression of FoxE3, a differentiation gene in the lens epithelium, showed a gradual reduction with age in the control lens, but a significant reduction after birth in the Rest CKO lens (Fig 7A). Although the expression of lens fiber-differentiation genes including c-Maf and Prox1 did not exhibit constant changes in the control lens, that of c-Maf was significantly
down-regulated in the Rest CKO lens (Fig 7B) while that of Prox1 was also down-regulated in the Rest CKO lens, except around birth (Fig 7C), suggesting that the cataract phenotype may be caused by changes in lens-differentiation gene expression. Reduction of Prox1 protein in the developing lens after E16.5 [38] may be corresponding to reduction of Prox1 mRNA observed after E17.5 to P1 (Fig 7C). Sharply FGF dependent expression of Prox1 protein in the lens fiber cells [39] may be related with the observed complex changes of Prox1 mRNA. The down-regulated expression of E-cadherin (CDH) and Mip was observed after P1 (Fig 7D and 7E). CDH was constantly expressed in the control, whereas that in Rest CKO lens was significantly down-regulated after birth (Fig 7D). On the other hand, the expression of Mip was gradually up-regulated in control with age, but in Rest CKO lens, Mip expression was significantly reduced in comparison with that of control mice after birth (Fig 7E). The expression of a series of gamma-crystallin genes was also slightly down-regulated in Rest CKO mice after P1 (Fig 7F–7J). Decreases in these genes required for the function and maintenance of lens fiber cells may have ultimately led to the formation of vacuole-like structures in the Rest CKO lens.

We used Sox1-Cre to knockout Rest, and this is a knockin mutation that destroys one allele of Sox1 [22] and may change Sox1-dependent gamma crystallin expression [28]. In order to exclude the possibility that Sox1 haploinsufficiency interferes with lens developmental studies or causes the observed reduction in gamma crystallin transcripts shown in Fig 7, we also analyzed mice with two alleles with or without Sox1-Cre allele (S4 Fig). A gene expression analysis using the lenses from Cre-positive controls and Rest CKO mice revealed that Rest expression levels in the lens were significantly lower in Rest CKO mice than in Cre-positive controls, whereas, Rest4 levels were not as described previously (S4A and S4B Fig). Cre expression was constantly high in mice with Sox1-Cre, irrespective of the Rest CKO allele while that in mice without Sox1-Cre was constantly low irrespective of the Rest CKO allele (S4C Fig). The expression levels of Rest target genes, such as Tubb3 and Syt4, were significantly higher in homozygous Rest2lox/2lox CKO mice than in Rest2lox/+ or Rest+/+ mice in the presence of Sox1-Cre allele. In the absence of Sox1-Cre allele, expression of these genes was rather constant in each Rest CKO allele (S4D and S4E Fig). The expression levels of Hes1 in lenses was also significantly higher in homozygous Rest2lox/2lox CKO mice than in Rest2lox/+ or Rest+/+ mice in the presence of Sox1-Cre allele (S4F Fig). The expression level of Sox2 in lenses was significantly higher in homozygous Rest2lox/2lox CKO mice at P0 (S4H Fig). No statistically significant differences of Sox1 expression was observed in each Rest CKO allele (S4G Fig). At P0, Sox1 expression levels were markedly lower in Rest2lox/2lox mice than in Rest+/+ mice for unknown reasons. We speculated that this may have been caused by the differences in the mouse line because we maintained the Sox1-Cre mouse line and Rest2lox/2lox mice line with and without the Sox1-Cre allele separately. While we cannot completely exclude the possibility that the phenotype we observed was the indirect, non-cell autonomous effect of Rest CKO including the changes of gene expression caused by the stress evoked by the vacuole formation, these results support conditional Rest ablation decreasing the genes related to lens differentiation and causing lens abnormalities.
Discussion

The REST repressor complex has been shown to regulate the differentiation of neuronal progenitors to mature neurons, during which the gradual loss of REST repressor complex binding with the target RE1 site ensures the up-regulated expression of target neuronal genes [7]. Although the embryonic lethal nature of \textit{Rest} gene knockout mice has hampered investigations on the roles of REST in the late developmental stage, we generated \textit{Rest} CKO mice and demonstrated that \textit{Rest} played a role in the differentiation and maturation of lens fiber cells.

Sox1-directed Cre expression started as early as E7.5 in the neural plate of the embryo and E10.5 in developing lens cells. Furthermore, although the expression of \textit{Rest} was detected in the developing lens by E13.5, a detectable change in \textit{Rest} CKO in the lens that was first observed around birth, suggesting that the indispensable function of REST in lens development was exerted in the later developmental stage. The eyes of all \textit{Rest} CKO mice developed severe lens opacity 8 weeks after birth and the rupture of the lens capsule is easily observed once removed from the eye. This defect in \textit{Rest} CKO mice appeared to be restricted to the lens structure, without any morphological malformation in other ocular tissues; however, further detailed analyses of gene and protein expression profiles and the distribution of each cell type constituting eye tissues are warranted. The first detectable symptom, a small vacuole-like gap in the posterior part of the lens around birth, was not preceded by an apoptotic reaction until the progression of symptoms to the adult.

A quantitative RT-PCR analysis suggested the activation of Notch signaling in the lens of \textit{Rest} CKO mice. A CtIP-PET analysis of REST target genes revealed the presence of REST target cis elements, named the RE1 site, inside or close to genes such as \textit{β-Catenin}, \textit{Hes1}, \textit{Jagged2}, and \textit{p21} [34], and the expression of all these genes was up-regulated in the \textit{Rest} CKO lens (S3G Fig, Fig 6K, S2C Fig, S3A Fig, respectively). Although the precise mechanisms underlying the increase in Notch signaling in the \textit{Rest} CKO lens requires further investigation based on a previous study that identified \textit{Hes1} as an upstream negative regulator of \textit{Rest} [40], REST may affect Notch signal-related genes by directly binding to their RE1 site. The involvement of Notch signaling was directly examined in the developing lens by mutated Notch signaling molecules as early as E11 [41]. However, in the present study, REST was responsible for a deleterious Notch signaling defect just after birth, suggesting the importance of Notch signaling even for the later stage of lens morphogenesis. It is important to note that the Sox1-Cre system also induces the deletion of \textit{Rest} in developing retinal tissues and this may indirectly influence the development of closely associated lens tissues. However, any possible defects caused by \textit{Rest} CKO in the retina, as is expected by the influence of Notch signaling in the maintenance of an retinal progenitor cell population and the proper development of retinal cells including photoreceptor and ganglion cells [42], was never observed in our \textit{Rest} CKO mice, indicating the lens cell-autonomous nature of the \textit{Rest} CKO phenotype.

Under the condition that Notch signaling is augmented by the targeted deletion of \textit{Rest}, reductions in \textit{FoxE3} gene expression regulating lens cell differentiation were observed in contrast to the reported increase after the activation of Notch signaling [41]. The up-regulated expression of \textit{FoxE3} was achieved under the constitutive activation of Notch signaling by the forced expression of NICD, which may have induced a markedly larger increase in Notch signaling than that induced by \textit{Rest} CKO in the present study. In any case, the expression of

\textbf{Fig 7. Gene expression of lens markers in various developmental stages of \textit{Rest} CKO mice.} The mRNA levels of each gene in lenses dissected from the various developing stages of \textit{Rest} CKO and their control mice were measured by quantitative RT-PCR. Transcript levels were normalized to β-actin levels. Data are presented as average values with s.d. of more than three to fifteen independent samples. *, p<0.05. **, p<0.01.

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FoxE3 was only down-regulated by 2-fold by Rest KO and this may be the reason that we did not detect impaired embryonic lens cell development. It is also important to note that RE1 sites have also been detected near the coding regions of the E-cadherin, Crystallin A, and Crystallin B genes [34], indicating that Rest is directly involved in lens fiber differentiation through the expression of these functional genes. Nevertheless, REST has a wide variety of target genes and may indirectly govern the necessary signaling pathway and each functional molecule in order to coordinate the proper cellular physiology required for functional lens fiber cells, and we observed changes in the expression of these candidates.

Since the cataract symptoms of Sox1-Cre+; Rest2lox/2lox mice starts just after birth and obvious lens opacification is detected as early as one week after birth, the Rest KO mouse is an uncommon cataract model. While cataracts have widely variable phenotypes [43], many of the genes responsible for congenital cataracts in humans are structural, such as the crystalline family and connexins or genes related to signal transduction. Although several transcription factors such as Pax6 [44], PITX3 [45], HSF4 [46], and HMX1 [47] were allelic with congenital cataracts, MAF family genes are well characterized for the indispensable transcription factor regulating lens development [48,49]. L-Maf/MafA is not required for normal mouse lens development [50], however, L-Maf/MafA and c-Maf are required for lens differentiation because they regulate crystallins [51,52]. Instead of increasing the number of lens epithelial cells by an aberrant cell specification process, the differentiation of lens fiber cells from precursor cells may be gradually impaired in Rest KO lens and ultimately manifest as a cataract phenotype.

In conclusion, we herein demonstrated that Rest KO mice showed a rare cataract phenotype, which severely affected lens differentiation. The deprivation of Rest from lens precursor cells caused a wide range of gene expression changes, including those for the Notch signaling pathway and lens differentiation and function, which eventually induced the unique cataract phenotype expressed from birth.

Supporting Information

S1 Fig. Immunohistochemical detection of lens marker proteins in Rest–deficient eyes in vivo. HE staining (A) and immunohistochemical staining with antibodies against Cryg (B), Prox1 (C), and c-Maf (D) at E15.5 (a and b), P0 (c and d), P7 (e and f), and 8W (g and h) in eyes from Rest KO mice (b, d, f, h) and their control littermates (a, c, e, g). HE staining showed significant disorganization after birth (A). Cryg was strongly expressed in both genotypes at all time points (B). The expression levels of Prox1 and c-Maf in lenses were lower in Rest KO mice than in the controls after birth (C and D). The scale bar represents 400 μm. (TIF)

S2 Fig. Expression of the control β-actin gene, genes related to Notch signaling, and lens-specification genes determined by quantitative RT-PCR. (A) β-actin expression for the quantitative control showed its constant expression in each developmental stage. (B-D) Expression of genes downstream of Notch signaling such as Jagged1, Jagged2, and Dll1 in the Rest-deficient lens. (E-G) Expression of genes related to lens-specification Pax6, Sox2 and Six3 in the Rest-deficient lens. Transcript levels were normalized to β-actin. Data are presented as average values with s.d. of more than three to fifteen independent samples. *: p<0.05, **: p<0.01. (TIF)

S3 Fig. Expression of cell cycle-related genes determined by quantitative RT-PCR. (A-F) Expression of cell cycle-related genes in various developmental stages. The expression of cyclin-dependent kinase-related genes, such as p21, p27, and p57, was significantly up-
regulated in the lenses of Rest CKO mice, whereas that of Cyclin D1 was not up-regulated. The expression of c-Myc and K-Ras was up-regulated in the lenses of Rest CKO mice. Transcript levels were normalized to the level of β-actin in each sample. Data are presented as average values with s.d. of more than three to fifteen independent samples. *: p<0.05, **: p<0.01.

(TIF)

S4 Fig. A gene expression analysis of the Rest-deficient lens and control lens having the Sox1-Cre transgene. The mRNA levels of Rest (A), Rest4 (B), Cre (C), Rest target genes, Tubb3 and Syt4 (D and E), Hes1 (F), Sox1 (G) and Sox2 (H) in Rest+/+, Restlox/+, and Restlox/lox allele with or without Sox1-Cre allele were measured by quantitative RT-PCR. +/+, 2lox/+, and 2lox/2lox indicate Rest+/+, Rest2lox/+, and Rest2lox/2lox allele, respectively. Cre+ indicates the presence of single Sox1-Cre allele and Cre- indicates the absence of Sox1-Cre allele. Transcript levels were normalized to β-actin. Data are presented as average values with s.d. of more than three to fifteen independent samples. *: p<0.05, **: p<0.01.

(TIF)

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Author Contributions

Conceptualization: HA.
Data curation: TK HO.
Formal analysis: HA.
Funding acquisition: HA AH TK.
Investigation: HA AH.
Methodology: HA HT.
Project administration: HA.
Resources: HA.
Supervision: TK.
Validation: HT AH.
Visualization: HA.
Writing – original draft: HA.
Writing – review & editing: AH TK HO.

References

1. Chong JA, Tapia-Ramirez J, Kim S, Toledo-Aral JJ, Zheng Y, Boutros MC, et al. REST: a mammalian silencer protein that restricts sodium channel gene expression to neurons. Cell 1995; 80: 949–957. PMID: 7697725
2. Schoenherr CJ, Anderson DJ. The neuron-restrictive silencer factor (NRSF): a coordinate repressor of multiple neuron-specific genes. Science 1995; 267: 1360–1363. PMID: 7871435

3. Ballas N, Battaglioli E, Atou F, Andres ME, Chenoweth J, Anderson ME, et al. Regulation of neuronal traits by a novel transcriptional complex. Neuron 2001; 31: 353–365. PMID: 11516394

4. Bruce AW, Donaldson IJ, Wood IC, Yerbury SA, Sadowski MJ, Chapman M, et al. Genome-wide analysis of repressor element 1 silencing transcription factor/neuron-restrictive silencing factor (REST/NRSF) target genes. Proc Natl Acad Sci USA 2004; 101: 10458–10463. PMID: 15240883

5. Johnson R, Gamblin RJ, Ooi L, Bruce AW, Donaldson IJ, Westhead DR, et al. Identification of the REST regulon reveals extensive transposable element-mediated binding site duplication. Nucleic Acids Res. 2006; 34: 3862–3877. PMID: 16899447

6. Otto SJ, McCorkle SR, Hover J, Concan C, Han JJ, Impey S, et al. A new binding motif for the transcriptional repressor REST uncovers large gene networks devoted to neuronal functions. J Neurosci. 2007; 27: 6729–6739. PMID: 17581960

7. Ballas N, Grunseich C, Lu DD, Sph JC, Mendel G. REST and its corepressors mediate plasticity of neuronal gene chromatin throughout neurogenesis. Cell. 2005; 121: 645–657. PMID: 15907476

8. Chen ZF, Paquette AJ, Anderson DJ. NRSF/REST is required in vivo for repression of multiple neuronal target genes during embryogenesis. Nat Genet. 1998; 20: 136–142. PMID: 9771705

9. Aoki H, Harra A, Era T, Kunisada T, Yamada Y. Genetic ablation of Rest leads to intraventricular derepression of neuronal genes during neurogenesis. Development. 2012; 139: 667–677. doi: 10.1242/dev.072272 PMID: 22241837

10. Kok FO, Taibi A, Wanner SJ, Xie X, Marave CE, Love CE, et al. Zebrafish REST regulates developmental gene expression but not neurogenesis. Development. 2012; 139: 3838–3848. PMID: 22951640

11. Abrajano JJ, Qureshi IA, Gokhan S, Zheng D, Bergman A, Meher M. Differential deployment of REST and CoREST promotes glial subtype specification and oligodendrocyte lineage maturation. PLoS One. 2009; 4: e7665. doi: 10.1371/journal.pone.0007665 PMID: 19883432

12. Kohyama J, Sanosaka T, Tokunaga A, Takatsuka E, Tsujimura K, Okano H, et al. BMP-induced REST regulates the establishment and maintenance of astrocytic identity. J Cell Biol. 2010; 189: 159–170. doi: 10.1083/jcb.20100420 PMID: 20351065

13. Aoki H, Harra A, Oomori Y, Shimizu Y, Yamada Y, Kunisada T. Neonatal lethality of neural crest cell-specific Rest knockout mice is associated with gastrointestinal distension caused by aberrations of myenteric plexus. Genes Cells. 2014; 19: 723–742. doi: 10.1111/gtc.12172 PMID: 25135772

14. Lu T, Aron L, Zullo J, Pan Y, Kim H, Chen Y, Yang, et al. REST and stress resistance in ageing and Alzheimer's disease. Nature. 2014; 507: 448–454. doi: 10.1038/nature13163 PMID: 24670762

15. Yang YJ, Baltus AE, Mathew RS, Murphy EA, Evrony GD, Gonzalez DM, et al. Microcephaly gene links trithorax and REST/NRSF to control neural stem cell proliferation and differentiation. Cell. 2012; 151: 1097–1112. doi: 10.1016/j.cell.2012.10.043 PMID: 23178126

16. Bassuk AG, Wallace RH, Buhr A, Bulter AR, Aafawi Z, Shimojo M, et al. A homozygous mutation in human PRICKLE1 causes an autosomal-recessive progressive myoclonus epilepsy-ataxia syndrome. Am J Hum Genet. 2008; 83: 572–581. doi: 10.1016/j.ajhg.2008.10.003 PMID: 18976727

17. Zuccato C, Belyaev N, Conforto P, Ooi L, Tartari M, Papadimou E, et al. Widespread disruption of repressor element-1 silencing transcription factor/neuron-restrictive silencer factor occupancy at its target genes in Huntington's disease. J Neurosci. 2007; 27: 6972–6983. PMID: 17596446

18. Canzonetta C, Mulligan C, Deutsch S, Rui F, O'Doherty A, Lyle R, et al. Dyrk1a-dosage imbalance perturbs NRSF/REST levels, deregulating pluripotency and embryonic stem cell fate in Down syndrome. Am J Hum Genet. 2008; 83: 388–400. doi: 10.1016/j.ajhg.2008.08.012 PMID: 18771760

19. Lepagnol-Bestel AM, Zvara A, Maussion G, Quignon F, Ngimbous B, Ramoz N, et al. Dyrk1a interacts with the REST/NRSF-SWI/SNF chromatin remodelling complex to deregulate gene clusters involved in the neuronal phenotypic traits of Down syndrome. Hum Mol Genet. 2009; 18: 1405–1414. doi: 10.1093/hmg/ddp047 PMID: 19218269

20. Ogino H, Yasuda K. Sequential activation of transcription factors in lens induction. Dev Growth Differ. 2000; 42: 437–448. PMID: 11041485

21. Yamada Y, Aoki H, Kunisada T, Harra A. Rest promotes the early differentiation of mouse ESCs but is not required for their maintenance. Cell Stem Cell. 2010; 6: 10–15. doi: 10.1016/j.stem.2009.12.003 PMID: 20065738

22. Takashima Y, Era T, Nakao K, Kondo S, Kasuga M, Smith AG, et al. Neuroepithelial cells supply an initial transient wave of MSC differentiation. Cell. 2007; 129: 1377–1388. PMID: 17604725

23. Srinivas S, Watanabe T, Lin CS, William CM, Tanabe Y, Jessell TM, et al. Cre reporter strain produced by targeted insertion of EFYFP and ECFP into the ROSA26 locus. BMC Dev Biol. 2001; 1: 4. PMID: 11299042
24. Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet. 1999; 21: 70–71. PMID: 9916792
25. Aoki H, Hara A, Motohashi T, Kunisada T. Keratinocyte stem cells but not melanocyte stem cells are the primary target for radiation-induced hair graying. J Invest Dermatol. 2013; 133: 2143–2151. doi: 10.1038/jid.2013.155 PMID: 23549419
26. Aoki H, Hara A, Motohashi T, Kunisada T. Protective effect of Kit signaling for melanocyte stem cells against radiation-induced genotoxic stress. J Invest Dermatol. 2011; 131: 1906–1915. doi: 10.1038/jid.2011.148 PMID: 21633369
27. Pavly LV, Sockanathan S, Placzek M, Lovell-Badge R. A role for SOX1 in neural determination. Development. 1998; 125: 1967–1978. PMID: 9550729
28. Nishiguchi S, Wood H, Kondoh H, Lovell-Badge R, Episkopou V. Sox1 directly regulates the γ-cristallin genes and is essential for lens development in mice. Genes Dev. 1998; 12: 776–781. PMID: 9512512
29. Johnson DS, Mortazavi A, Myers RM, Wold B. Genome-wide mapping of in vivo protein-DNA interactions. Science. 2007; 316: 1497–1502. PMID: 17540862
30. Johnson R, Teht CH, Kunarso G, Wong KY, Kondoh H, Lovell-Badge R, et al. REST regulates distinct transcriptional networks in embryonic and neural stem cells. PLoS Biol. 2008; 6: e256. doi: 10.1371/journal.pbio.0060256 PMID: 18959480
31. Rowan S, Conley KW, Le TT, Donner AL, Brown NL. Notch signaling regulates growth and differentiation in the mammalian lens. Dev Biol. 2008; 321: 111–122. doi: 10.1016/j.ydbio.2008.06.002 PMID: 18588711
32. Le TT, Conley KW, Mead TJ, Rowan S, Yutzey KE, Brown NL. Requirements for Jag1- Rbpj mediated Notch signaling during early mouse lens development. Dev Dyn. 2012; 24: 493–504.
33. Ogino H, Ochi H, Reza HM, Yasuda K. Transcription factors involved in lens development from the pre-placodal ectoderm. Dev Biol. 2012; 363: 333–347. doi: 10.1016/j.ydbio.2012.01.006 PMID: 22269169
34. Duncan MK, Cui W, Oh DJ, Tomarev SI. Prox1 is differentially localized during lens development. Mech Dev. 2002; 112: 195–198. PMID: 11850194
35. Audette DS, Anand D, So T, Rubenstein TB, Lachke SA, Lovicu FJ, et al. Prox1 and fibroblast growth factor receptors form a novel regulatory loop controlling lens fiber differentiation and gene expression. Development. 2016; 143: 318–328. doi: 10.1242/dev.127860 PMID: 26657765
36. Abderrahmani A, Niederhauser G, Lenain V, Regazzi R, Waeger G. The hairy and enhancer of split 1 is a negative regulator of the repressor element silencer transcription factor. FEBS Lett. 2005; 579: 6199–6204. PMID: 16253247
37. Ochi H, Fisher M, Grainger RM. Convergence of a head-field selector Otx2 and Notch signaling: a mechanism for lens specification. Development. 2008; 135: 249–258. PMID: 18057103
38. Riesenberg AN, Liu Z, Kopan R, Brown NL. Rbpj Cell autonomous regulation of retinal ganglion cell and cone photoreceptor fates in the mouse retina. J Neurosci. 2009; 29: 12865–12877. doi: 10.1523/JNEUROSCI.3382-09.2009 PMID: 19828801
39. Francis PJ, Berry V, Moore AT, Bhattacharya S. Lens biology: development and human cataractogenesis. Trends Genet. 1999; 15; 191–196. PMID: 10322486
40. Glaser T, Jepeal L, Edwards JG, Young SR, Favor J, Maas RL. PAX6 gene dosage effect in a family with congenital cataracts, aniridia, anophthalmia and central nervous system defects. Nat Genet. 1994; 7: 463–471. PMID: 7951315
41. Berry V, Francis PJ, Prescott Q, Waseem NH, Moore AT, Bhattacharya SS. A novel 1-bp deletion in PITX3 causing congenital posterior polar cataract. Mol Vis. 2011; 17: 1249–1253. PMID: 21633712
42. Merath K, Ronchetti A, Sidjian DJ. Functional analysis of HSF4 mutations found in patients with autosomal recessive congenital cataracts. Invest. Ophthalmol. Vis Sci. 2013; 54: 6646–6654. doi: 10.1167/iovs.13-12283 PMID: 24045990
47. Gillespie RL, Urquhart J, Lovell SC, Biswas S, Parry NR, Schorderet DF, et al. Abrogation of HMX1 function causes rare oculoauricular syndrome associated with congenital cataract, anterior segment dysgenesis, and retinal dystrophy. Invest Ophthalmol Vis Sci. 2015; 56: 883–891. doi: 10.1167/iovs.14-15861 PMID: 25574057

48. Deng H, Yuan L. Molecular genetics of congenital nuclear cataract. Eur J Med Genet. 2014; 57: 113–122. doi: 10.1016/j.ejmg.2013.12.006 PMID: 24384146

49. Hejtmancik JF. Congenital cataracts and their molecular genetics. Semin Cell Dev Biol. 2008; 19: 134–149. PMID: 18035564

50. Takeuchi T, Kudo T, Ogata K, Hamada M, Nakamura M, Kito K, et al. Neither MafA/L-Maf nor MafB is essential for lens development in mice. Genes Cells. 2009; 14: 941–947. doi: 10.1111/j.1365-2443.2009.01321.x PMID: 19624757

51. Kawauchi S, Takahashi S, Nakajima O, Ogino H, Morita M, Nishizawa M, et al. Regulation of lens fiber cell differentiation by transcription factor c-Maf. J Biol Chem. 1999; 274: 19254–19260. PMID: 10383433

52. Ogino H, Yasuda K. Induction of lens differentiation by activation of a bZIP transcription factor, L-Maf. Science. 1998; 280: 115–118. PMID: 9525857