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Targeted deletion of Dicer disrupts lens morphogenesis, corneal epithelium stratification, and whole eye development

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Abstract

Dicer, a ribonuclease essential for miRNA processing, is expressed abundantly in developing mouse cornea and lens. We studied the roles of Dicer and miRNAs in eye development by conditionally deleting the *Dicer* gene in the mouse lens and corneal epithelium. Adult *Dicer* conditional null (*Dicer*CN) mice had severe microphthalmia with no discernible lens and a poorly stratified corneal epithelium. Targeted deletion of *Dicer* effectively inhibited miRNA processing in the developing lens at 12.5 day of embryogenesis (E12.5). Lens development initiated normally but underwent progressive dystrophy between E14.5 and E18.5. Microarray analysis revealed activation of P53 signaling in *Dicer*CN lenses at E13.5, consistent with increased apoptosis and reduced cell proliferation between E12.5 and E14.5. Expression of Pax6 and other lens developmental transcription factors were not greatly affected between E12.5 and E14.5 but decreased as the lens degenerated. Our data indicated an indispensable role for Dicer and miRNAs in lens and corneal development.

Keywords

Dicer; microRNA; lens development

INTRODUCTION

The mammalian eye is a complex neurosensory organ. Development of a functional eye requires a series of interactions between different tissues (Lang, 2004; Cvekl and Duncan, 2007). Our interest has focused on the development of lens and cornea (Piatigorsky, 1998; Swamynathan et al., 2008). In brief, mouse eye development begins at 8.5 days of embryogenesis (E8.5) when the evaginating optic vesicle from the forebrain induces the lens placode which subsequently invaginates and separates from the surface ectoderm to form a lens vesicle. By E14.5, the anterior epithelial cells of the lens vesicle proliferate and form a cubical epithelium; the posterior cells of the lens vesicle stop dividing and differentiate into the primary non-nucleated fiber cells (Lang, 1999; Bhat, 2001). The ectodermal epithelial cells overlying the lens vesicle are destined to become the corneal epithelium (Zieske, 2004). At E12.5 the developing corneal epithelium becomes bilayered and neural crest cells start to migrate into the space between the developing lens and presumptive corneal epithelium, giving rise to the corneal endothelium and the stromal keratocytes. The corneal epithelium remains only 2–3 cell layers at postnatal day 10 (PN10). Stratification and maturation of the corneal epithelium coincides with eye opening two weeks after birth. By six weeks after birth, the corneal epithelium reaches 6–8 cell layers (Hay, 1979) and is

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composed of flattened cells at the surface and posterior columnar cells. At the molecular level, differentiation of the lens and cornea requires sequential activation of multiple transcription factors (e.g. Pax6, Prox1, and Pitx3 for lens development; Pax6, Hes1 and Klf4 for corneal development), as well as interactions between the transcription factors and signaling pathways from certain growth factors (e.g. Bmp and Fgf) (for reviews, see Ogino and Yasuda, 2000; Lang, 2004; Cvekl and Duncan, 2007).

microRNAs (miRNAs) are single-stranded, noncoding RNAs of 17–25 nucleotides. miRNA genes are transcribed by the RNA polymerase II (Pol II) as primary miRNAs (pri-miRNAs) (Lee et al., 2004). The pri-miRNAs are sequentially processed by two RNaseIII enzymes, Drosha and Dicer, to give rise to approximately 22 nucleotide-long double-stranded duplexes. The duplexes are incorporated into RISC (RNA-induced silencing complex), where the mature miRNA strands are directed to mRNA targets by sequence complementarity to the 3'-untranslated regions of the mRNAs (Lewis et al., 2005). miRNAs inhibit the expression of the mRNA targets in one of two ways, either by the cleavage and degradation of the mRNAs (Bagga et al., 2005; Lim et al., 2005), or inhibition of mRNA translation and expression (Gregory et al., 2005). Estimates indicate that miRNAs repress expression of more than 30% of the protein-coding genes at the posttranscriptional level in mammals. miRNAs can also lead to stimulation of mRNA translation (Pillai et al., 2005) and control of DNA transcription (Kim et al., 2008).

Hundreds of miRNA genes, mostly evolutionarily conserved, have been identified. Some are expressed in many tissues (cf let-7 family members), while others are expressed in a strict cell/tissue-specific pattern (cf muscle-specific *miR-1* and *-208*) (Zhao et al., 2007). miRNA are key regulators in various aspects of tissue development and homeostasis (Kusenda et al., 2006; Zhao and Srivastava, 2007), including differentiation, apoptosis, proliferation, and the maintenance of cell and tissue identity during embryogenesis and adult life. miRNA expression has also been linked to disease. For example, aberrant expression of *miR-21* has been associated with tumor initiation and progression (Shi et al., 2008). In particular, several miRNAs have been identified that are highly enriched in mouse cornea and lens (Ryan et al., 2006; Karali et al., 2007). Many of these corneal/lens miRNAs display spatial and temporal specificity, raising the question of how miRNAs function in cornea and lens development. Interestingly, a recent study has shown that suppression of lipid phosphatase SHIP2 expression by one of the corneal miRNAs, *miR-205*, can be antagonized by another corneal-enriched miRNA, *miR-184* (Yu et al., 2008), suggesting complicated roles for miRNAs in regulation of ocular differentiation.

In the present investigation we investigated the general roles of miRNAs in corneal and lens development by focusing on Dicer, the essential ribonuclease for miRNA maturation. Our results reveal abundant expressions of Dicer and miRNAs during corneal and lens development. We created a conditional deletion of *Dicer* in the developing lens placode and presumptive corneal epithelium by using the Cre-LoxP technology (Ashery-Padan et al., 2000; Murchison et al., 2005). Our results reveal a critical role for Dicer in regulation of apoptosis and cell proliferation in the developing lens and cornea, and establish the importance of miRNAs expressed in the lens and cornea for proper development on the whole eye.

RESULTS

***Dicer* and miRNA Expression in the Developing Lens and Cornea**

We first examined Dicer and miRNA expression during cornea and lens development in the mouse. As lens morphogenesis mainly occurs during embryonic development while cornea maturation is not complete until six weeks after birth, we examined Dicer expression in the

developing mouse lens at E12.5 and developing mouse cornea at postnatal day (PN) 9; we also examined the expression in the mature lens and cornea of a 6-week-old wild type mouse. qRT-PCR analysis revealed that *Dicer* expression in the PN9 cornea was comparable to that in the mature cornea, which was 7 times higher than that in the mature heart (Chen et al., 2008; da Costa Martins et al., 2008) (Figure 1). By contrast, *Dicer* expression in the developing lens at E12.5 was about 3 times higher than in the mature lens of 6-week-old mice; *Dicer* expression in the mature lens was comparable to that in the mature heart. The decreased expression of *Dicer* transcripts in the mature lens reflects the non-nucleated, transcriptionally silent state of a majority of the mature lens fiber cells (Zelenka, 2004; Lovicu and McAvoy, 2005). In conclusion, the qRT-PCR assay demonstrated abundant *Dicer* expression in both the developing lenses and corneas.

We next determined miRNA profiles in the wild type mouse cornea at PN9 before stratification of the corneal epithelium and at 6 weeks of age when the epithelium is fully stratified. Using Sanger miRBase Version 10.0, we analyzed 568 miRNAs across 4 biological replicates of the developing (PN9) and mature (6-week-old) mouse cornea. The microarray data have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE16209 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE16396>). Abundant miRNAs were expressed in both the PN9 cornea and the mature 6-week-old cornea. Table 1A lists 10 miRNAs that gave the strongest hybridization signal in the 6-week mature cornea. In particular, *miR-184* was highly enriched in both the developing and mature corneas and gave the highest hybridization signal of all the corneal miRNAs at both developmental stages. While *Dicer* expression levels were similar in the PN9 and 6-week-old cornea, the expression profiles of miRNAs were very different between the two developmental stages. 78 of the 568 (~13.7%) miRNAs showed differential expression between the PN9 and 6-week-old mouse corneas, with *P* values being <0.05. In particular, members of the miR-29 family, *miR-29b* and *miR-29c*, were up-regulated by more than 80-fold in the 6-week-old cornea. Table 1B lists the miRNAs showing the highest fold change between the two developmental stages. Abundant *Dicer* expression in the developing lens and cornea, as well as a dynamic change in miRNA expression during corneal maturation, suggests a critical role for *Dicer* and miRNAs in lens and cornea development.

Conditional Knockout of *Dicer* in the Lens/Cornea Leads to Microphthalmia

To explore the role of *Dicer* and miRNAs in cornea and lens development, we generated a cornea/lens-specific deletion of the *Dicer* gene. We used an established *Dicer^{loxP/loxP}* mouse line in which the essential exons encoding the two RNaseIII domains are flanked by *loxP* sites (Murchison et al., 2005). *Dicer^{loxP/loxP}* mice were crossed with *Le-Cre* transgenic mice, where Cre recombinase and green fluorescent protein (GFP) are expressed via the *Pax6 ectoderm enhancer (EE)*. *Le-Cre* is able to drive efficient deletion of target genes at the surface ectoderm-derived lens and cornea as early as E9.5 (Ashery-Padan et al., 2000). The resultant *Dicer* conditional null (*Dicer*CN) mice were viable and fertile. However, as the *Le-Cre* also directs *Dicer* deletion in the pancreas (Ashery-Padan et al., 2000), the *Dicer*CN mice have a short life span (less than 6 months), possibly due to low levels of blood insulin (0.1±0.05 ng/ml vs. 0.49±0.22 ng/ml in controls) and high levels of blood glucose (986±138 mg/dl vs. 151±15 mg/dl in controls).

6-month-old *Dicer^{loxP/loxP}* mice without the *Cre* gene showed normal ocular morphology (Figure 2A) as did mice harboring the *Cre* transgene alone (*Le-Cre; Dicer^{+/+}*) or *Cre* with the heterozygous floxed *Dicer* (*Le-Cre; Dicer^{+/loxP}*) (data not shown). However, age-matched *Le-Cre; Dicer^{loxP/LoxP}* mice (*Dicer*CN) were microphthalmic (Figure 2D). The eyelids were closed (not fused) and covered the entire eye. The beneath eyeballs were less than one tenth the size of the wild type control mice (Figure 2E). To study the morphology

of the microphthalmic eyeballs, serial sagittal sections were cut through the whole eye. Figure 2F showed that a representative mid-plane section of *Dicer*CN eyes without lens. The corneal epithelium was thinner and less stratified in the *Dicer*CN mice (Figure 2F1), as compared to 6–8 layers of corneal epithelium in the age-matched controls (Figure 2C1). The basal layer of the *Dicer*CN corneal epithelium appeared flattened in contrast to the columnar epithelium of the control cornea and large vacuoles were detected in the corneal epithelium of the *Dicer*CN mice (arrowheads in Figure 2F). Although *Dicer* was deleted only in the developing corneal and lens epithelia, defects were observed throughout other ocular tissues. For example, the corneal stroma was thinner and the keratinocytes in the stroma had irregularly round- or star-shaped appearance (Figure 2F1). The iris and ciliary body were poorly developed. The iris without pupil opening is adherent to the cornea, the anterior chamber angle was absent, and the anterior chamber was flat. The inner retina was folded and disorganized. The sclera was poorly identifiable. Similar microphthalmic phenotypes have been observed in the PN3 and 3-month-old *Dicer*CN mice (data not shown).

Eye Development in *Dicer*CN Mice

We next investigated whether the aphakia (lack of lens) of the adult *Dicer*CN mice resulted from the failure to initiate lens specification, or from progressive lens dystrophy at later development. Inspection of the embryo revealed that the structure and morphology of the developing eye appeared normal at E12.5 in the *Dicer*CN mice (Figure 3E). The lens vesicle pinched off from the surface ectoderm and the primary fiber cells started to form normally. The elongating lens primary fiber cells filled the lumen of the lens vesicle at E14.5 (Figure 3F). A distinct lens equatorial region was formed in *Dicer*CN mice by E14.5, but the *Dicer*CN lens appeared slightly smaller relative to the wild type lens and the lens epithelium contained fewer cells compared to the controls. There were no detectable histological abnormalities in cornea and retina at E14.5 in the *Dicer*CN mice.

By E16.5 the lens fiber cells were prominently dystrophic and the lens epithelium was not visible in the *Dicer*CN mice (Figure 3G). Lens dystrophy continued at E18.5 (Figure 3H), leading to aphakia in the newborns (data not shown). The retinal pigmented epithelium at E18.5 extended to the anterior region of the eye and there was no discernible differentiation of ciliary body and iris (Figure 3H). In the control wild type embryos, protrusions and migration of the primitive eyelids proceeded normally and by E18.5, the eyes were completely covered with fused eyelids (data not shown). In contrast, migration of the primitive eyelids halted by E18.5 in *Dicer*CN mice (Figure 3H) and eyelid fusion was never observed in the mutants during embryonic development (data not shown). Although Cre simultaneously deletes *Dicer* expression in the developing lens and cornea epithelium (Ashery-Padan et al., 2000), the morphology and integrity of the corneal epithelium appeared normal by E18.5, in contrast to the marked dystrophy of the lens at the same age.

We assessed *Dicer* inactivation and the consequent inhibition of miRNA maturation in the *Dicer*CN mice during embryonic development. We performed Taqman[®] microRNA real-time PCR assays, which only detect the levels of mature miRNAs (Chen et al., 2005). Compared to the expression in controls, *miR-184*, a cornea/lens-specific miRNA (Ryan et al., 2006; Karali et al., 2007), was reduced by 85% in the *Dicer*CN lens vesicles at E12.5, and more than 90% at E14.5 (Figure 3I). Significant reductions were also observed in expressions of other mature miRNAs as well as expression of functional *Dicer* transcripts at E12.5 (data not shown). Le-Cre transgenic mice start to express Cre recombinase in the prospective lens cells and corneal epithelial cells as early as E9.5 (Ashery-Padan et al., 2000; Miller et al., 2006). Therefore, the residual amount of functional miRNAs and *Dicer* mRNAs at E12.5 in the *Dicer*CN lenses suggests a short delay between activation of Cre recombinase and effective ablations of all functional *Dicer* and mature miRNAs, a phenomenon that has also been observed in other tissues and cells where *Dicer* was

inactivated (Andl et al., 2006; O'Rourke et al., 2007). This delay may arise from the long half-life of *Dicer* mRNA and/or miRNAs; alternatively, it may have resulted from incomplete Cre-mediated recombination.

***Dicer* Deletion Altered the Expression Profile of Protein-Coding Genes in Developing Lenses**

In order to investigate the mechanism for the progressive lens dystrophy in *Dicer*CN eyes, we compared expression profiles of protein-coding genes in wild type and *Dicer*CN lenses at E13.5, at a time before gross morphological changes had occurred. Using the Affymetrix mouse gene 1.0 ST array, we analyzed gene expressions across five biological replicates of the wild type and mutant lenses at E13.5. The microarray data have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE16209 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE16209>). Of the 28,853 identified transcripts, 1165 showed differential expression (accumulated FDR<0.05) between the wild type and the *Dicer*CN lenses. 315 transcripts were up-regulated while 850 were down-regulated in the *Dicer*CN lenses relative to the control lenses. Table 2 lists the top 10 most differentially expressed transcripts between the wild type and *Dicer*CN lenses. Some of these differentially expressed genes may be direct targets for miRNAs, while expressions of others may be indirectly affected by the loss of miRNA expression. Using the terms from gene ontology, we studied the common characteristics among the differential expressed genes. Various subunits of the ribosomal complex (20 transcripts) were consistently down-regulated in the *Dicer*CN lenses (data not shown), suggesting severely impaired machinery for protein biosynthesis in the absence of *Dicer* and miRNAs.

Amongst the differentially expressed genes, there is a significant enrichment for the genes involved in apoptosis and cell death (Figure S1). In particular, many genes implicated in P53 signaling pathways, including *Tp53inp1*, *Tnfrsf10A*, *Serpine2*, and *Tp53* itself, were up-regulated by at least 1.5-fold in the *Dicer*CN lenses at E13.5 compared to that in the controls (Table 3). Many genes involved in cell cycle progression were also differentially expressed (Table 4), suggesting a de-regulation of cell cycle in *Dicer*CN lenses at E13.5. Severe disruption of cell cycle control may lead to cessation of cell growth and induction of apoptosis in the *Dicer*CN lenses as shown with other knockout mouse models (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992; Nagahama et al., 2001).

Gene set enrichment analysis also showed that a significant portion of the differentially expressed genes was involved in tissue specification and organ development (Figure S1 and Table S3). In particular, genes involved in epithelial cell differentiation, including *Fzd1* and *Cdh1*, were differentially expressed in the *Dicer*CN lenses. A few genes important for lens specification were down-regulated in the *Dicer*CN lenses (Table 5). Lens crystallin genes, including $\beta A2$ -, $\beta A4$ -, γN -, and γS -crystallins, were down-regulated in the *Dicer*CN lenses, with the expression of the lens-specific γS -crystallin (Sinha et al., 1998) down-regulated by more than 10-fold. It is noteworthy, however, that unlike β - and γ -crystallin gene expression, α -crystallin gene expression was not affected by the targeted deletion of *Dicer*. Also down-regulated in the *Dicer*CN lenses were *Fgfr2* and *Cdh1* (*E-Cadherin*). Targeted deletion of these two genes in the lens is associated with impaired lens development and microphthalmia in adult eyes (Garcia et al., 2005; Pontoriero et al., 2008b).

Decreased Cell Proliferation and Increased Cell Death in *Dicer*CN Lens and Cornea

Inasmuch as the microarray data showed activation of apoptosis signaling pathway along with inhibition of cell growth pathway in the *Dicer*CN lenses at E13.5, we investigated whether lens dystrophy in *Dicer*CN mice was a result of aberrant cell proliferation and/or

cell death. Proliferation of the lens epithelia was significantly reduced as early as E12.5 (Figure 4B). The BrdU labeling index of the control lens was about 33%, whereas 16% of the *Dicer*CN lens cells were BrdU-positive ($P=0.02$; Figure 4G). By E14.5, lens proliferation was almost abolished in the lens epithelia of the *Dicer*CN mice as judged by the BrdU incorporation, while the lens epithelia of the controls mice remained proliferative at the same embryonic stages (data not shown).

TUNEL-staining revealed increased apoptosis in the mutant lens early during embryonic development. *Dicer*CN lenses consistently showed a substantial increase of TUNEL-positive cells already at E12.5 (Figure 4D). Although the number of the total nuclei observed was comparable ($p>0.1$) in the control (277 ± 91) and *Dicer*CN (257 ± 60) lenses at E12.5, 11% of the *Dicer*CN lens nuclei were TUNEL-positive nuclei while only 0.7% of the control lenses nuclei contained TUNEL-positive nuclei ($p<0.01$; Figure 4G). Many of the TUNEL-positive cells were located in the epithelium of the *Dicer*CN lenses, as well as in the equatorial region (arrows in Figure 4D). Consistent with increased apoptosis, active caspase-3 was detected in the *Dicer*CN lens as early as E12.5 (Figure 4F), but not in the wild type lens (Figure 4E). As a result of the increased apoptosis and decreased cell proliferation, the number of total nuclei in the *Dicer*CN lenses (207 ± 31) was significantly reduced ($p<0.02$) as compared to that in the control lenses (380 ± 41) by E14.5. The percentage of the TUNEL-positive lens cells was also higher in the E14.5 *Dicer*CN lenses (8%) than in the control lenses (0.4%). The continued increase in apoptosis and cessation of cell proliferation at E14.5 accounted for the prominent lens atrophy observed between E14.5 to E18.5 in the *Dicer*CN embryos.

Expressions of Critical Developmental Transcription Factors in the *Dicer*CN Mice

Microarray data suggested that mRNA levels of transcription factors critical for lens development, including *Pax6*, *Prox1*, *Pitx3*, and *c-Maf*, were not significantly changed in *Dicer*CN lenses at E13.5 (data not shown). Also, based on immunostaining of the embryonic sections, *Pax6*, *Prox1*, and *Pitx3* expressions were comparable in *Dicer*CN and control lenses between E12.5 and E13.5 (Figure 5). There were overall fewer *Pax6* positive cells in the E14.5 *Dicer*CN lens (Figure 6B), consistent with the progression of apoptosis and lens dystrophy. Nonetheless, *Pax6* staining in the remaining lens epithelial cells at E14.5 appeared similar to that in the wild type lens cells. *Pax6* staining was gradually lost between E16.5 and E18.5 as the *Dicer*CN lenses continued to degenerate (Figures 6D and 6F). In contrast to the loss of *Pax6* expression in the lens by E18.5, *Pax6* expression appeared similar in the corneal epithelium of the *Dicer*CN and wild type mice at this stage.

DISCUSSION

The present results establish an indispensable role for *Dicer* in the development of the lens and cornea in the mouse. As *Dicer* primarily catalyzes miRNA maturation in mammalian species (Ambros et al., 2003; Lippman and Martienssen, 2004; Calabrese et al., 2007; Babiarz et al., 2008), our study implicates miRNAs in key aspects of lens and cornea development. Although *Dicer* was recently implicated in the biogenesis of endogenous siRNAs in mouse oocytes (Tam et al., 2008; Watanabe et al., 2008), sequencing of small RNA species from *Dicer*-null stem cells (Calabrese et al., 2007) has suggested that defects observed in *Dicer* deletion result from the loss of miRNAs rather than other small RNA species.

The progressive lens dystrophy in the *Dicer*CN embryonic mice was accompanied by microphthalmia and supports the idea that the lens is a developmental organizer for the anterior segment of the eye (Coulombre, 1969; Beebe and Coats, 2000; Yamamoto and Jeffery, 2000; Kurita et al., 2003; Strickler et al., 2007). The severe microphthalmia in

*Dicer*CN mice resembled that in transgenic mice in which the lenses were ablated by lens-specific expression of a diphtheria toxin transgene (Zhang et al., 2007). The diphtheria toxin-induced microphthalmic eyes had a poorly differentiated ciliary body and iris, folded retina, and no significant anterior chamber. In contrast to the major abnormalities in the anterior regions of the *Dicer*CN eyes, the retina of the *Dicer*CN mice, although convoluted, was laminated and had differentiated cell layers in the newborns. We did observe, however, increased cell death in the *Dicer*CN retina at E16.5 (data not shown) suggesting a role for lens on retinal cell survival, particularly the inner retina (Strickler et al., 2007).

The developing eyelids failed to migrate and fuse during embryogenesis, causing the defect of eye-open at birth (EOB) in the *Dicer*CN mice. It has been shown that eyelid fusion has a protective role for the developing eye, particularly the cornea (Teramoto et al., 1988). Thus, failure of eyelid fusion may affect corneal maturation in the *Dicer*CN mice. On the other hand, development of the corneal epithelium was less affected than that of the lens despite simultaneous deletion of the floxed *Dicer* in both tissues. The corneal epithelia of the adult *Dicer*CN mice, however, were poorly stratified and displayed large vacuoles. At present, it is unclear whether the defect in stratification of the corneal epithelium is due to an autonomous role of *Dicer* and miRNA in corneal development or whether it is a secondary effect caused by lens dystrophy and/or eyelid defects.

Increased apoptosis in *Dicer*CN lenses, along with decreased cell proliferation, resulted in massive lens dystrophy between E14.5 to E18.5. Microarray analysis of differential gene expression in the *Dicer*CN lenses at E13.5 showed that many genes involved in the P53 pathway were up-regulated. Among them, *Tp53inp1*, *Cdkn1a* (*p21Cip*), *Serpine2*, and *Tnfrsf10A* are the key regulators of cell death and cell growth arrest (Okamura et al., 2001; Baetu and Hiscott, 2002; Jackson and Pereira-Smith, 2006; Kortlever and Bernards, 2006). Furthermore, phenotypes of the *Dicer*CN lenses resembled the P53-dependent lens dystrophy in *Creb2*-deficient mice (Tanaka et al., 1998; Hettmann et al., 2000). In both cases, the initial stages of lens development including formation of the optic vesicle and primary lens fibers elongation occurred normally. However, at around E14.5 lens epithelial cells underwent massive apoptosis, causing aphakia in the newborns. Our data thus suggest that activation of the P53 signaling pathway may play an important role in lens dystrophy induced by *Dicer* and miRNA deletion. Indeed, recent studies have revealed involvement of multiple miRNAs in the P53 network, either as downstream effectors (He et al., 2007), or upstream regulators of P53 and/or its modifiers (Rane et al., 2009).

Lens development requires sequential activation of multiple transcription factors and growth factors signaling pathways. Our microarray and immunostaining studies suggest that, upon global removal of miRNAs, expression levels of lens transcription factors (*Pax6*, *Prox1*, *Pitx3*, *Foxe3*, *Tcfap2a*, and *Sox2*) known to direct lens development (Ogino and Yasuda, 2000; Cvekl and Duncan, 2007) were not significantly affected (either directly or indirectly) before lens dystrophy. The progressive lens dystrophy in *Dicer*CN mice contrasts with the microphthalmic phenotypes caused by deletions of one of the aforementioned transcription factors. For example, defects in lens vesicle formation was detected as early as E10.5 when *Pax6* (Ashery-Padan et al., 2000) was conditionally knocked out using the same Le-Cre transgenic line as used in the *Dicer*CN mice. In addition, deletion of *Foxe3* (Miller et al., 2006) or *Tcfap2a* (Miller et al., 2006; Pontoriero et al., 2008a) resulted in defective separation of the lens vesicle from the surface ectoderm between E9 and E10. Loss of *Pax6* expression at later developmental stages was coincided with progressive lens dystrophy. It is possible that targeted *Dicer* deletion in the present study affected *Pax6* expression at a later developmental stage due to relatively long half-lives of the involved mature miRNAs. Alternatively, our observations may suggest that loss of the critical transcription factors in the *Dicer*CN lens was a consequence, rather than a cause, of lens dystrophy; and that

regulation of lens development by miRNAs operates downstream from the sequential activation of lens-specific transcription factors.

Our microarray data raise the possibility that reduced expression of β - and γ -crystallin genes contributes to lens dystrophy in the *Dicer*CN mice. γ -crystallins especially are lens-specific (Sinha et al., 1998) and γ S-crystallin, which is highly enriched in lens fiber cells, has been associated with lens dystrophy and cataract (Sun et al., 2005). *Fgfr2* expression is also down-regulated in the *Dicer*CN lenses. FGF signaling has been implicated in lens induction (Faber et al., 2001) and the differentiation of lens fiber cells (Lang, 1999). *Fgfr2* is required for lens fiber differentiation (Robinson, 2006). *Fgfr2* conditional null lenses show increased apoptosis by E12.5, as do *Dicer*CN lenses, and degenerate after birth (Garcia et al., 2005). The phenotypic similarities between the *Dicer* and *Fgfr2* conditional knockout mice suggest that regulation of *Fgfr2* levels in the developing lenses may be a pathway through which *Dicer* and miRNAs promote lens development.

Finally, although speculative, it may be of interest to consider the possibility that some miRNAs contribute to lens differentiation by suppressing expression of protein-coding genes that direct non-ocular cell fates. *Itgb8* is an example of an up-regulated gene in *Dicer*CN lenses that is implicated in vascular morphogenesis rather than lens differentiation (Zhu et al., 2002). Other up-regulated genes in *Dicer*CN lenses involved in the development of various tissues are *Eya1* (ear and kidney) (Xu et al., 1999), *Klf-10* (bone morphogenesis) (Subramaniam et al., 2005; Hawse et al., 2008), *Prkcd* (immune tolerance) (Miyamoto et al., 2002) and *Pik3cg* (thymocyte development) (Sasaki et al., 2000). The functions of *miR-133* in mesoderm development (Ivey et al., 2008) and of *miR-196* in limb development (Hornstein et al., 2005) represent examples of this “fail-safe” mechanism of suppressing the expression of genes that are not appropriate for development of a specific tissue. Thus, we suggest that lens miRNAs may serve at a secondary level to limit the expression of genes that could interfere with proper lens development. Identification of miRNA target genes will be instrumental for further investigations of the roles for the many miRNAs that are dynamically expressed in the developing lens and cornea.

Experimental Procedures

Conditional disruption of *Dicer*—The *Le-Cre* mice in which the Pax6 P0 promoter drives the expression of Cre recombinase in the cornea, lens, and endocrine pancreas from E9.0 onward (Ashery-Padan et al., 2000) were mated to the mice that had LoxP sites inserted in the introns downstream of exon 21 and exon 23 of the *Dicer* gene (Murchison et al., 2005). The heterozygous progeny were crossed to obtain *Le-Cre*^{-/-}; *Dicer*^{loxP/loxP} (*Dicer*CN) and *Dicer*^{loxP/loxP} (control) offsprings. Matings were set to ensure that only one copy of the transgene Cre was present, as homozygous *Le-Cre* can sometimes cause lens phenotype (Garcia et al., 2005). Genomic DNA isolated from tail clippings of these mice was assayed for the presence of the *Dicer-LoxP* (Murchison et al., 2005) and *Le-Cre* transgenes (Ashery-Padan et al., 2000) by PCR using specific primers. Mice studied here were on a mixed genetic background and maintained in accordance with the guidelines set forth by the Animal Care and Use Committee of the National Eye Institute, NIH.

Histology and Immunohistochemistry—Enucleated eyeballs or heads of the staged embryos were fixed in 10% buffered formalin, embedded in paraffin, and 5 μ m sections were cut. For morphological studies, the slides were stained with hematoxylin and eosin (H&E; Poly Scientific, Bayshore, NY). Antibody staining was performed on deparaffinized sections after inactivation of the endogenous peroxidase activity with 0.3% H₂O₂ for 30 min. Antigen retrieval was performed in citrate buffer (pH 6.0) using a pressure cooker (Electron Microscopy Sciences, PA). For color development in immunohistochemistry, we used a

biotinylated secondary antibody from the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA), followed by incubation with diaminobenzidine (DAB; Vector Laboratories). For immuno-detection in immunofluorescence, we applied on the sections Alexa-fluor-568-labeled anti-rabbit antibody (Invitrogen, Carlsbad, CA) following primary antibody incubation. For cell proliferation studies on the embryonic eyes, pregnant female mice were injected intraperitoneally with 100 µg 5-bromo-2'-deoxyuridine (BrdU) per gram of body weight and sacrificed 4–6 hours later. Heads of the staged embryos were fixed, embedded in paraffin and sectioned. Deparaffinized sections were incubated with a monoclonal anti-BrdU antibody, followed by a color reaction using a Vectastain Elite ABC kit as described above. Apoptosis was analyzed via in situ labeling of DNA fragments using an ApopTag Red In Situ kit (Millipore, Billirica, MA) according to the manufacturer's instructions. Sections were then counterstained either with DAPI (4', 6'-diamidino-2-phenylindole) using Prolong Gold anti-fade reagent (Invitrogen, Carlsbad, CA) or hematoxylin.

For the quantification of BrdU-positive cells and TUNEL-positive nuclei at E12.5 and E14.5 lenses, at least three sections from each of the two control and two *Dicer*CN embryos were counted. Percentages of the positive cells were calculated as the number of BrdU (or TUNEL)-positive cells divided by total numbers of nuclei for each section.

The following antibodies were used in this study: rat anti-BrdU (1:200; Catalog #: OBT0030G, Accurate Chemical and Scientific, Westbury, NY); rabbit anti-Pax6 (1:5000; Catalog #: PRB-278P, Covance, Vienna, VA); rabbit anti-Prox1 (1:10000; Catalog #: PRB-238C, Chemicon, Temecula, CA); rabbit anti-active caspase-3 (1:200; Catalog #: 9661, Cell Signaling Technology, Danvers, MA); rabbit anti-Pitx3 (1:200; Catalog #: 38-2850, Invitrogen, Carlsbad, CA).

Isolation of total RNA, real time qRT-PCR, and microarray analyses—Intact lenses were dissected from five control and five *Dicer*CN mice at E13.5 and immediately snap-frozen in liquid nitrogen. The pair of lenses from each mouse was combined for isolation of total RNAs so that five independent samples for each group (control versus *Dicer*CN lenses) were submitted for gene expression analysis. Microarray analysis was performed at Expression Analysis (Durham, NC). After confirming the RNA quality with bioanalyzer, 100 ng total RNAs per sample were labeled and hybridized with mouse gene 1.0 ST arrays from Affymetrix (Santa Clara, CA). The microarray data have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE16209 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE16209>). Expression profiling of the E13.5 control and *Dicer*CN lenses was compared by a Permutation Analysis for Differential Expression (PADE). The False Discovery Rate (FDR) was calculated for each potential differentially expressed transcript. Calculation of fold change was based on the control group relative to the *Dicer*CN group, with a negative value indicating expression reduction in the *Dicer*CN group relative to the controls. Differentially expressed transcripts were selected according to the following criteria: (1) the accumulated FDR was less than 0.05 (maximal p-value for the selected transcripts was less than 0.01), and (2) the absolute fold change of the average expression signal from the two groups was greater than 1.3 between the control and *Dicer*CN. Data were analyzed through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com, Ingenuity Systems Inc, Redwood City, CA).

Corneas were dissected from C57BL/6 mice sacrificed at PN9 and 6 weeks (adult) after birth. Total RNAs were isolated from ten 6-week and sixteen PN9 whole corneas. Cornea dissection and RNA extraction for both the 6-week and PN9 corneas were repeated four

times to collect altogether eight RNA samples (four adult cornea RNAs and four PN9 cornea RNAs). The samples were sent to LC Sciences LLC (Houston, TX) for miRNA microarray assay. All probe sequences in the array were based on Sanger miRBase Release 10.0. The signals were presented after background subtraction, normalization and detection evaluation. Statistic significance was set by P-value < 0.05. The microarray data have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE16209 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE16396>).

miRNA expression levels were examined using the TaqMan® MicroRNA Assay Kit (Applied Biosystems, Foster City, CA). The kit uses gene-specific stem-loop reverse transcription primers and TaqMan probes to detect only mature miRNA transcripts in a two-step qRT-PCR assay. Either U6 snRNA or 18S rRNA was used as an internal control for the normalization in the Taqman microRNA assay. There were no significant differences in the outcome when using different internal controls. *Dicer* mRNA levels were examined via SYBR Green real time qRT-PCR using either GAPDH or 18S rRNA as the internal control. The primer pair for *Dicer* used in the PCR step is as follows: 5'-AAG GGC AGA GCG CAA GTC AGT CA-3' and 5'-ACA CAC GCC TCC TAC CAC TAC AAC AC-3'. The two primers reside at exon 22 and exon 23, respectively, which are flanked by the loxP sites in the *Dicer^{loxP/loxP}* mice (Murchison et al., 2005). Thus, conditional excision of the fragment between the loxP sites by the Cre recombinase removed the template for the primer pair in the *Dicer* mRNAs, and therefore no product is predicted in the qRT-PCR assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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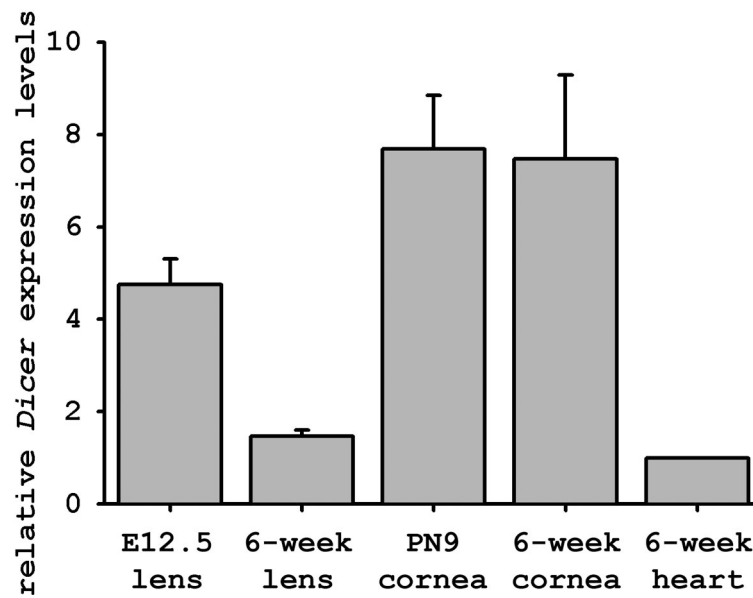


Figure 1.

Expressions of *Dicer* during cornea and lens development of a wild type mouse. qRT-PCR was used to quantify *Dicer* mRNA levels in lenses and corneas at various developmental stages relative to the *Dicer* expression level in the 6-weeks-old mouse heart, which was arbitrarily set to 1. Data shown were obtained from three isolations of total RNA analyzed in triplicate. Error bars represent the standard deviation between experiments.

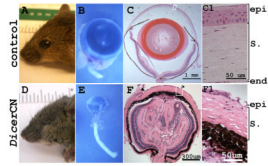


Figure 2.

Targeted deletion of *Dicer* in lens and cornea caused microphthalmia in *DicerCN* adult mice. The eyeballs were enucleated from a 6-month-old *DicerCN* mouse (B) and its age-matched wild type control (A). A typical mid-plane cross-section (C and F) showed lack of lens (F) in the conditional knockout. Close investigation of the eye structure showed that the corneal epithelium (F1) in the *DicerCN* mice was stratified into 3–4 layers while the epithelium in the control mice was stratified into 6–8 layers (C1). Abbreviations: epi: corneal epithelium; S.: corneal stroma; end: corneal endothelium.

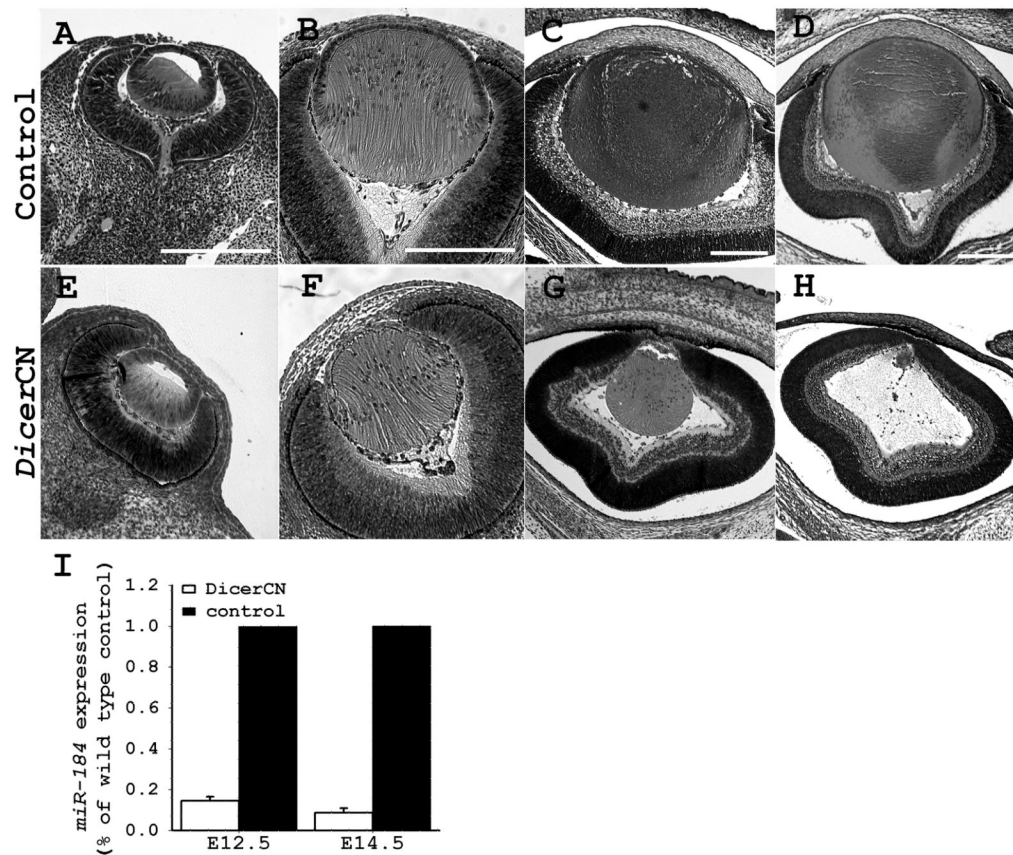


Figure 3. Comparison of lens morphogenesis between *DicerCN* and control mice. (A–D): development of control lenses. (E–H): development of *DicerCN* lenses. (A and E): lenses at E12.5; (B and F): lenses at E14.5; (C and G): lenses at E16.5; (D and H): lenses at E18.5. Scale bar=200 μ m. (I). qRT-PCR analysis of the developing lenses showed that expression of *miR-184* was significantly reduced in the *DicerCN* lenses at E12.5 and E14.5. Data shown were obtained from three isolations of total RNA analyzed in triplicate with each primer pair. Error bars represent the standard deviation between experiments.

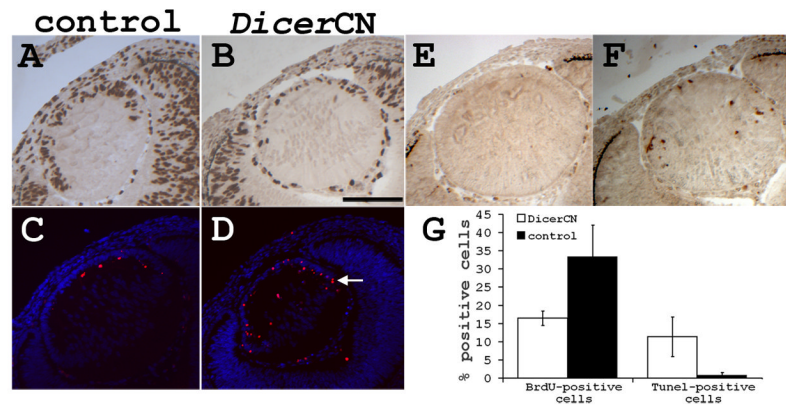


Figure 4. *DicerCN* lenses had increased apoptosis and reduced proliferation at E12.5. (A, C, and E): control lenses. (B, D, and F): *DicerCN* lenses. (A–B): BrdU staining. (C–D): TUNEL staining (red) imposed on nuclear DAPI staining (purple). (E–F): active caspase-3 staining (red). Scale bar=150 μ m. White arrow marks the equatorial region of a developing lens. (G) Quantification of BrdU-positive cells and TUNEL-positive nuclei in control and *DicerCN* littermate lenses. At least three sections from each of the two control and two *DicerCN* embryos were counted.

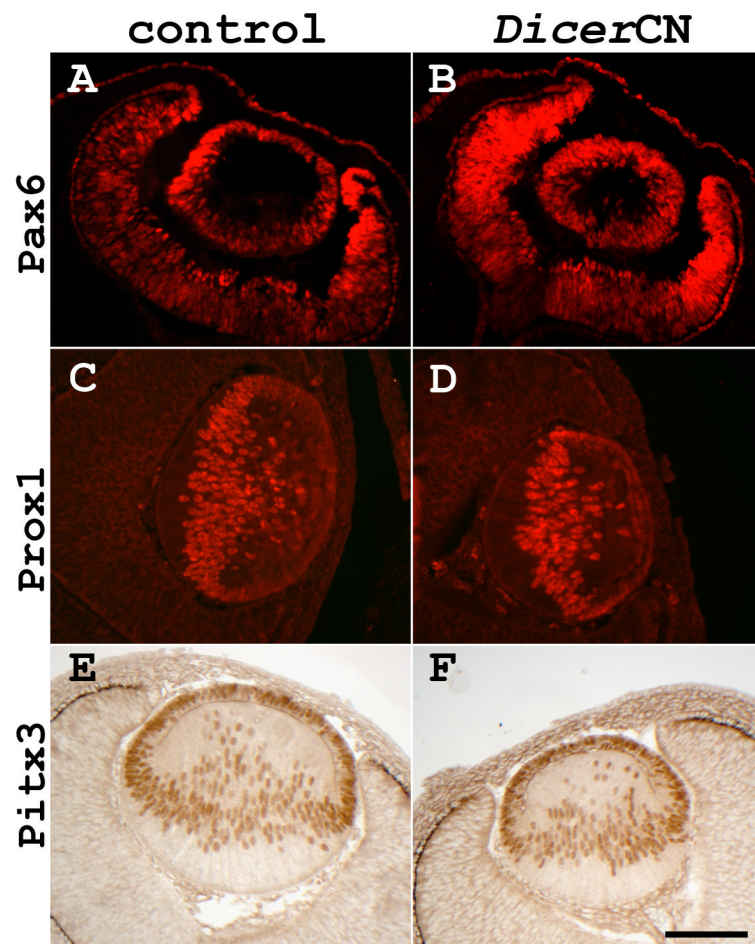


Figure 5. Immunostaining of critical transcription factors for lens specification. (A, C, and E): control lenses. (B, D, and F): *DicerCN* lenses. (A–B): anti-Pax6 staining at E12.5; (C–D): anti-Prox1 staining at E13.5; (E–F): anti-Pitx3 staining at E13.5. Scale bar=100 μ m

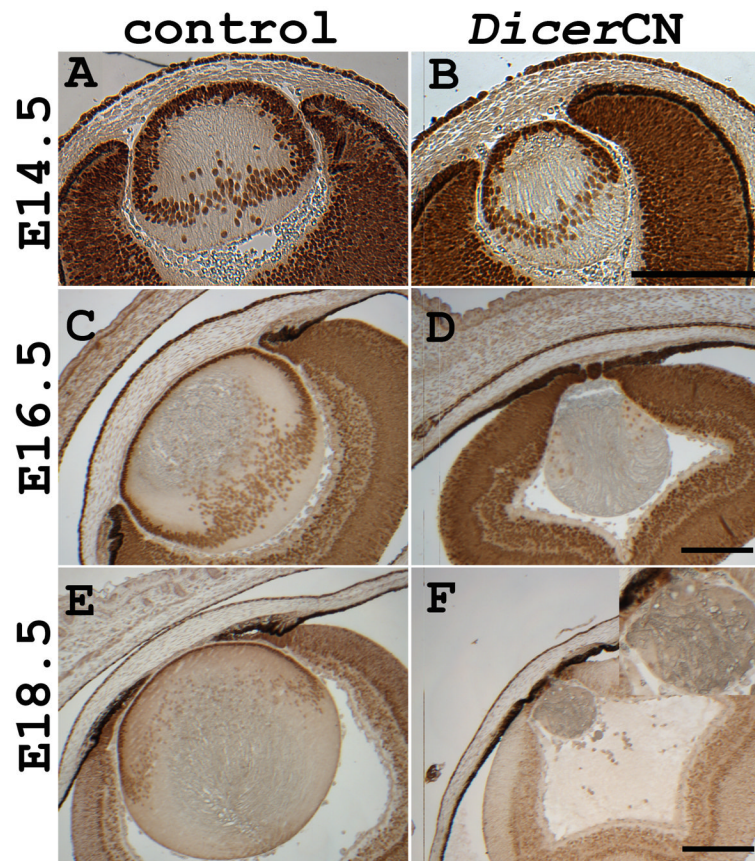


Figure 6. Pax6 expression decreased at later developmental stages, coincident with lens dystrophy. Expression of Pax6 was examined using anti-Pax6 antibody at E14.5 (A–B), E16.5 (C–D), and E18.5 (E–F). (A, C, and E): control lenses; (B, D, and F): *DicerCN* lenses. Inset in Figure F shows the degenerative *DicerCN* lens at a higher magnification. Scale bar=200 μm

Table 1

miRNA expression profiles in mouse corneal development

Table 1A. Most abundant miRNAs in 6-week-old mouse cornea	Table 1B. Differential expressed miRNAs between PN9 and 6-wk cornea	
	miRNA	fold change (6wk:PN9)^I
<i>mmu-miR-184</i>	<i>mmu-miR-29b</i>	471.01
<i>mmu-miR-205</i>	<i>mmu-miR-29c</i>	81.56
<i>mmu-miR-26a</i>	<i>mmu-miR-31*</i>	61.26
<i>mmu-miR-125b-5p</i>	<i>mmu-miR-141</i>	38.29
<i>mmu-miR-23b</i>	<i>mmu-miR-31</i>	36.85
<i>mmu-miR-23a</i>	<i>mmu-miR-434-3p</i>	-32.10
<i>mmu-miR-709</i>	<i>mmu-miR-101a</i>	31.58
<i>mmu-let-7a</i>	<i>mmu-miR-96</i>	31.37
<i>mmu-miR-204</i>	<i>mmu-miR-29a</i>	25.64
<i>mmu-let-7f</i>	<i>mmu-miR-382</i>	-22.22

^I fold change is base on expression in 6-wk cornea relative to that in PN9, with negative values indicating expression decrease in 6-wk cornea compared to PN9 cornea.

Table 2The most differentially expressed genes between the *Dicer*CN and the control lenses at E13.5

Unigene	Gene Description	Fold ^I	Gene function
Mm.6253	Crygs (crystallin, gamma S)	-10.24	lens development
Mm.45494	Gcg (glucagon)	-7.52	insulin secretion
Mm.95780	Lrtm1 (leucine-rich repeats and transmembrane domains 1)	-5.11	---
Mm.4258	Ogn (osteoglycin)	-4.21	---
Mm.127156	Prosl (protein S, alpha)	3.47	blood coagulation
Mm.200608	Clu (clusterin)	-3.46	cell death
Mm.392646	Btg2 (B-cell translocation gene 2)	3.30	transcription
Mm.260988	Slc7a11 (solute carrier family 7, member 11)	-3.29	amino acid transportation
Mm.4159	Thbs1 (thrombospondin 1)	3.22	angiogenesis
Mm.405761	Igfbp5 (insulin-like growth factor binding protein 5)	-3.20	cell growth

^I fold change is based on expressions in the *Dicer*CN lenses relative to that in the control lenses, with negative value indicating expression decrease in the *Dicer*CN compared to the control lenses.

Table 3

differentially expressed genes affiliated with P53 signaling pathway

Unigene	Gene Description	Fold^I
Mm.4159	Thbs1 (thrombospondin 1)	3.22
Mm.253819	Pik3r3	2.92
Mm.393018	Tp53inp1 (p53 inducible nuclear protein 1)	2.79
Mm.195663	Cdkn1A (p21, Cip1)	2.62
Mm.3093	Serpine2 (PAI-1)	2.18
Mm.101369	Pik3cg	1.96
Mm.193430	Tnfrsf10A (TrailR1)	1.95
Mm.273049	Ccnd1 (Cyclin D1)	1.79
Mm.275071	Jun	1.63
Mm.222	Tp53 (tumor protein p53)	1.49
Mm.235194	Akt3	1.33
Mm.220289	Apaf1	1.30

^I fold change is based on expressions in *Dicer*CN lenses relative to that in the control lenses.

Table 4

differentially expressed genes affecting cell cycle progression

Unigene	Gene Description	Fold ¹
Mm.222178	Prkca (protein kinase C, alpha)	-2.18
Mm.2444	Myc	-2.01
Mm.35605	Cdh1 (E-cadherin) ²	-1.73
Mm.168789	Cdkn1c (p57, Kip2)	-1.37
Mm.307932	E2F2 (E2F transcription factor 2)	1.37
Mm.289662	Ddx3x (DEAD/H box polypeptide 3, X-linked)	1.37
Mm.222	Tp53 (tumor protein p53)	1.49
Mm.275071	Jun	1.63
Mm.2314	Prkcd (protein kinase C, delta)	1.74
Mm.273049	Ccnd1 (cyclin D1)	1.79
Mm.193099	FN1 (fibronectin 1)	1.87
Mm.195663	Cdkn1a (p21, Cip1)	2.62

¹ fold change is based on expressions in the *DicerCN* lenses relative to that in the control lenses, with negative value indicating expression decrease in the *DicerCN* compared to the control lenses.

² grey fonts mark the genes inhibiting while black fonts mark genes promoting cell cycle progression.

Table 5

Differential Expressed Genes Associated with Lens Development

Unigene	Gene Description	Fold¹
Mm.6253	Crygs (crystallin, gamma S)	-10.24
Mm.63484	Crygn (crystallin, gamma N)	-2.08
Mm.35605	Cdh1 (E-cadherin)	-1.73
Mm.86656	Cryba2 (crystallin, beta A2)	-1.7
Mm.475710	Fktn (fukutin)	-1.66
Mm.16340	Fgfr2 (fibroblast growth factor receptor 2)	-1.57
Mm.1090	Gpx1 (glutathione peroxidase 1)	-1.52
Mm.221403	Pdgfra	-1.5
Mm.18213	Tgfb2 (transforming growth factor, beta 2)	-1.42
Mm.168789	Cdkn1C (p57, Kip2)	-1.37
Mm.40324	Cryba4 (crystallin, beta A4)	-1.31
Mm.272321	Cited2	-1.31
Mm.268003	Insr (insulin receptor)	1.41

¹ negative values indicate expression decrease in the *Dicer*CN compared to the control lenses.