Roles for Dicer1 in the patterning and differentiation of the optic cup neuroepithelium

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SUMMARY
The embryonic ocular neuroepithelium generates a myriad of cell types, including the neuroretina, the pigmented epithelium, the ciliary and iris epithelia, and the iris smooth muscles. As in other regions of the developing nervous system, the generation of these various cell types requires a coordinated sequence of patterning, specification and differentiation events. We investigated the roles of microRNAs (miRNAs) in the development of optic cup (OC)-derived structures. We inactivated Dicer1, a key mediator of miRNA biosynthesis, within the OC in overlapping yet distinct spatiotemporal patterns. Ablation of Dicer1 in the inner layer of the OC resulted in patterning alteration, particularly at the most distal margins. Following loss of Dicer1, this region generated a cryptic population of cells with a mixed phenotype of neuronal and ciliary body (CB) progenitors. Notably, inactivation of Dicer1 in the retina further resulted in abrogated neurogenesis, with prolongation of ganglion cell birth and arrested differentiation of other neuronal subtypes, including amacrine and photoreceptor cells. These alterations were accompanied by changes in the expression of Notch and Hedgehog signaling components, indicating the sensitivity of the pathways to miRNA activity. Moreover, this study revealed the requirement of miRNAs for morphogenesis of the iris and for the regulation of CB cell type proliferation and differentiation. Together, analysis of the three genetic models revealed novel, stage-dependent roles for miRNAs in the development of the ocular sub-organs, which are all essential for normal vision.

KEY WORDS: Dicer1, MicroRNAs, Iris, Ciliary body, Optic cup, Retinogenesis, Mouse

INTRODUCTION
Normal vision depends on differentiation of the optic neuroepithelium into neuronal and non-neuronal ocular sub-organs: the neuroretina, retinal pigmented epithelium (RPE), the ciliary body (CB) and the iris. The neuroretina is populated by six classes of neuron and Müller glia that are organized in three cell layers. The outer nuclear layer (ONL) contains the cone and rod photoreceptors, whereas the inner nuclear layer (INL) is populated by the bipolar cells, horizontal cells, amacrine cells (ACs) and Müller glia. Finally, residing in the ganglion cell layer (GCL) are the displaced ACs and ganglion cells (GCs). Adjacent to the photoreceptor layer is the RPE, a cuboidal epithelial layer of pigmented cells essential for the maintenance and function of the photoreceptors (Strauss, 2005). Anterior to the retina and RPE are the ocular structures of the anterior segment of the eye: the CB and the iris. The CB is connected to the lens through zonular fibers and is composed of two epithelial layers that are tightly associated: a posterior non-pigmented layer and anterior pigmented layer. A primary role for the CB is secretion of the aqueous humor, and of components of the vitreous and inner limiting membranes. The CB is therefore pivotal to the establishment and maintenance of ocular pressure and to the survival of the GCs (Gould et al., 2004; Halfter et al., 2008; Halfter et al., 2005). The iris is composed of two layers of pigmented epithelium, muscles and stroma (for a review, see Davis-Silberman and Ashery-Padan, 2008). The iris controls the amount of light entering the eye and is involved in the circulation of the aqueous humor (Cvekl and Tamm, 2004; Davis-Silberman and Ashery-Padan, 2008).

Normal development of these structures provides a model system with which to investigate early patterning, gradual specification and subsequent differentiation into the mature cell types (Yang, 2004; Esteve and Bovolenta, 2006). FGFs secreted from the surface ectoderm and TGFβ from the surrounding ocular mesenchyme specify the RPE and neuroretina domains in the outer and inner layers of the optic cup (OC). The interface between the RPE and the neuroretina is the region that gives rise to the anterior structures. The inner layer of the OC contains the retinal progenitor cells (RPCs), which differentiate into the retinal cell types in a defined spatiotemporal order. GCs, cone photoreceptors and horizontal cells are formed first, followed by ACs and rod photoreceptors, with bipolar and Müller cells appearing last (Young, 1985).

The generation of neuronal and non-neuronal cell types from the OC is spatially and temporally regulated by intrinsic and extrinsic cues. Wnt and BMP signaling promote the non-neuronal fates, whereas Shh and Notch1 are involved in neurogenesis (Jadhav et al., 2006a; Jadhav et al., 2006b; Liu et al., 2007; Yaron et al., 2006; Yu et al., 2006; Zhao et al., 2002). Differentiation of the RPE and retina follows a center to periphery pattern, progressing towards the OC periphery and arresting at the distal-most tips, where the progenitors of the CB and iris reside (Davis-Silberman and Ashery-Padan, 2008).

Formation of the retina and anterior structures of the iris and CB depends on tight spatiotemporal regulation of gene expression. MicroRNAs (miRNAs) are small non-coding RNAs, which have emerged as key regulators of developmental events across phyla (Lee and Ambros, 2001; Pasquinielli et al., 2000; Reinhart et al., 2000). It has been suggested that most vertebrate genes are
regulated by miRNAs (Friedman et al., 2009b). miRNAs control gene expression post-transcriptionally by regulating either the stability or translation efficiency, or both, of different miRNAs. A single miRNA might directly alter the expression of hundreds of proteins to a mild extent, and indirectly affect the expression of thousands more (Baek et al., 2008; Selbach et al., 2008). Although the effect on the levels of individual proteins might be subtle, the summed effect is significant. Indeed, particular miRNAs have been linked to the formation of specific organs, demonstrating their crucial role during development (Maatouk and Harfe, 2006).

Primary miRNA transcripts are processed by two RNaseIII ribonucleases, Drosha and Dicer1 (Grishok et al., 2001; Giraldes et al., 2006), to form an ~22-bp miRNA duplex that is incorporated into the RNA-induced silencing complex (RISC). Although Dicer1 mutant mice die on embryonic day (E) 7.5 (Bernstein et al., 2003), a conditional inactivation approach enabled a study of its role in various organs, exposing the role of miRNAs in multiple developmental processes (e.g. Harfe et al., 2005; Harris et al., 2006).

Dozens of miRNAs have been detected in the mouse eye, some showing dynamic expression patterns during ocular development (Hackler et al., 2010; Xu et al., 2007). Targeting of Dicer1 in Xenopus embryos revealed its importance for cell-cycle exit, survival and differentiation timing of retinal cells (Decembrini et al., 2008). In mammals, conditional mutagenesis of Dicer1 was established using Chx10-Cre, which is active in a mosaic pattern in the retina but not in the non-neuronal progenitors of the CB and iris. The phenotype of Dicer1loxP/loxP;Chx10-Cre mutants included reduced survival of photoreceptors and improper lamination of the retina (Damiani et al., 2008). In a recent study, Dicer1 activity was explored in the inner layer of the OC by employing the a-Cre transgene (Georgi and Reh, 2010). That study revealed important roles for Dicer1 in the generation and survival of all retinal cell types. These intriguing data encouraged us to explore miRNA involvement in the patterning of the OC, in the generation of the derived non-neuronal structures and in the signaling pathways that might mediate the pleiotropic functions of miRNAs in the retina and the adjacent ocular sub-organs.

MATERIALS AND METHODS

Mice

The transgenic mouse lines Typr2-Cre, Z/AP, α-Cre, Pou4f3-Cre and Dicer1loxP/loxP were established as described previously (Davis et al., 2009; Lobe et al., 1999; Marquardt et al., 2001; Sage et al., 2006; Harfe et al., 2005) and Z/AP mouse lines were established as described previously (Davis et al., 2009; Lobe et al., 1999; Marquardt et al., 2001; Sage et al., 2006). Genotyping was performed by PCR using the primers listed in Table S1 in the supplementary material. All procedures involving animals followed NIH guidelines and were approved by the Animal Care and Use Committee of Tel Aviv University.

The day on which the copulatory plug was observed was defined as E0.5, and the day of birth was referred to as postnatal day 1 (P1). For pulse-chase experiments, pregnant females were injected with BrdU (140 µg/g body weight) and embryos or eyes were harvested at the specified ages. Specimens were fixed in 4% paraformaldehyde at 4°C, dehydrated in a graded ethanol series, and embedded in paraffin wax. Alternatively, fixed tissues were cryoprotected in 30% sucrose and embedded in OCT. For histological staining, 10 µm paraffin sections were stained with Hematoxylin and Eosin (H&E) according to a standard protocol. Alkaline phosphatase (AP) staining was performed on either 10 µm paraffin or 14 µm frozen sections as described previously (Lobe et al., 1999). For immunofluorescence analysis, either paraffin or frozen sections were stained as described by Marquardt et al. (Marquardt et al., 2001). Antibodies used were hAP (1:100, Santa Cruz), Pax6 (1:400, Convance), Cx43 (1:1000, Sigma), Cav3 (1:100, Santa Cruz), PCNA (1:100, Dako), Ki67 (1:100, Dako), CcnD1 (1:250, Thermo Scientific), Cav3D3 (1:100, Santa Cruz), p57kip2 (1:50, Santa Cruz), Pou4F2 (1:100, Santa Cruz), Rho (1:1000, generous gift from M. Applebury, Harvard Medical School, Boston, MA, USA), NF165 (1:500, Developmental Studies Hybridoma Bank), PKCoα (1:1200, Santa Cruz), syntaxin 1 (1:500, Sigma), Crlbp (1:1000, generous gift from J. C. Saari, University of Washington, Seattle, WA, USA), Sox2 (1:500, Chemicon), PH3 (1:500, Santa Cruz), Isl1 (1:100, Developmental Studies Hybridoma Bank), BrDU (1:100, Chemicon), Chx10 (1:1000, Exalpha), Mitf1 (1:1000, generous gift from K. Bharti, NINDS, Bethesda, MD, USA), cc53 (1:300, Cell Signaling), PNA (1:200, Vector Laboratories). For in situ hybridization, frozen sections were stained with DIG- or POD-labeled probes as reported by Yaron et al. (Yaron et al., 2006). Probes used were Otx1 and Bmp4 (generous gift from P. Bovolenta, Instituto Cajal, Madrid, Spain), Notch1 (Schroder and Gossler, 2002), Hes5 (Ohtsuka et al., 1999), Neurod1 (Lee et al., 1995), Pit1 (generous gift from C. L. Cepko, Harvard Medical School, Boston, MA), Crx (Furukawa et al., 1997), Foxa4 (generous gift from M. Xiang, Robert Wood Johnson Medical School, Piscataway, NJ, USA) and Gli1 (Furimsky and Wallace, 2006). Each experiment included at least three different animals from each genotype. For in situ hybridization of miR-204 we used LNA-probe (Exiqon), following the manufacturer’s instructions but with a minor modification as described by Xu et al. (Xu et al., 2007).

Measurements and morphometric analyses

To obtain position-matched sections, eyes were embedded parallel to the optic nerve and transverse serial sections were collected. For quantification of GCs in the neuroretinal layer (LNL), Pou4f2+ or Isl1+ cells in the Ki67+ region were counted and normalized to area. At least four sections per animal were averaged. Recombinant regions were defined using the hAP reporter gene. To assess the thickness of the GCL, we measured its area and divided it by overall retinal width.

RESULTS

Roles for miRNAs in development of the iris and the CB

The neuroretina and most of the iris and CB cell types originate from adjacent progenitors of the OC. To explore the potential role of miRNAs in the formation of these structures, we employed three Cre-transgenic lines, each with unique but partly overlapping activity in OC progenitors. The pattern of Cre activity was monitored by AP expression from the Z/AP reporter transgene (Fig. 1A,F,K; Fig. 2A,F) (Marquardt et al., 2001; Davis et al., 2009; Davis-Silberman et al., 2005; Lobe et al., 1999). The recombination activity of Typr2-Cre is initiated on around E11 in the ocular pigmented cell types (Davis et al., 2009). At late embryogenesis, it is expressed in the presumptive pigmented epithelia of the CB and the iris (Fig. 1A,F), and then in the muscles and stroma of the iris (Davis et al., 2009). The α-Cre line is active within the inner layer of the OC from E10.5, including in the progenitors of the neuroretina, the non-pigmented CB and the iris (Marquardt et al., 2001). Later, α-Cre activity overlaps with that of Typr2-Cre in the iris pigmented epithelia (IPE) and muscles (Fig. 1A,F,K) (Davis-Silberman et al., 2005). Finally, Pou4f3-Cre is instrumental for conditional mutagenesis in postnatal development of the anterior ocular structures, owing to its ectopic expression in the non-pigmented CB and the posterior IPE starting from P1 (Fig. 2A,F). At later stages, it is expressed in iris muscle as well (not shown). In contrast to the endogenous expression of Pou4f3, we did not detect Cre activity in the GCL.

Mutant mice from all genotypes displayed a variety of abnormalities. Dicer1loxP/loxP;Typr2-Cre adult mice showed poor survival and small body size, probably owing to Typr2-Cre expression in the developing forebrain and dorsal root ganglia (Davis et al., 2009). Similarly, most Dicer1loxP/loxP;Pou4f3-Cre
mutants died soon after birth, possibly due to a malformation of the upper jaw that impedes normal suckling (Friedman et al., 2009a). Mutant eyes of both genotypes, but not of Dicer1loxP/loxP;α-Cre mice, showed severe microphthalmia and lacked the vitreous cavity (Fig. 1G,L; data not shown). We next examined morphogenesis of the anterior structures of the mutants. First, we compared the formation of the CB in Dicer1loxP/loxP;Tyrp2-Cre and Dicer1loxP/loxP;α-Cre mutants. The recombination pattern of the Tyrp2-Cre transgene included the pigmented CB, whereas the α-Cre transgene was active in the non-pigmented layer (Fig. 1A). Nevertheless, the CB of both mutants was flattened and lacked the characteristic undulating processes (Fig. 1B,G,H,L). To verify the identity of the hypoplastic CB, we determined the expression of Pax6 and Cx43. After birth, Pax6 is normally expressed in retinal, iris and CB cell types, but not in the RPE, whereas Cx43 is highly expressed in the CB (Coffey et al., 2002; Walther and Gruss, 1991) (Fig. 1D,E). Both Cx43 and Pax6 were detected in the Dicer1loxP/loxP;Tyrp2-Cre and Dicer1loxP/loxP;α-Cre CBs (Fig. 1I,J,N,O), suggesting that the initial specification occurred but later morphogenesis was abrogated. These findings reveal an important role for miRNA in CB development.

In contrast to the comparable phenotype of the mutant CBs of these two mouse lines, the iris reacted differently. Both Cre lines were active within the IPE (Fig. 1A); however, the Tyrp2-Cre transgene was also expressed in the iris stroma (Davis et al., 2009). The main defect exhibited by the P8 Dicer1loxP/loxP;α-Cre iris was detachment of the two epithelial layers (Fig. 1M). Taken together, we concluded that miRNAs in the stroma are pivotal for iris formation, whereas their existence in the iris epithelia mainly contributes to cell-cell adhesion.

In both the α-Cre- and Tyrp2-Cre-mediated deletions of Dicer1, miRNA excision takes place at the progenitor stage, which potentially prevents the analysis of their late role in CB and iris differentiation. We therefore used the Pou4f3-Cre line, expressed postnatally in the anterior structures (Fig. 2A,F). Similar to Dicer1loxP/loxP;α-Cre line, the IPE was detached (Fig. 2G), although the sphincter muscle, marked with the specific antibody caveolin3 (Cav3) (Kogo et al., 2006), exhibited a distinctive overgrowth phenotype (Fig. 2G,H, compare with control Fig. 2B,C). The CB was hyperplastic and disorganized, and the two epithelial layers were detached (Fig. 2I,J, compare with control Fig. 2D,E). Hyperplasia might result from increased proliferation; indeed, Ki67+ cells were detected in the detached non-pigmented CB (Fig. 2J), which was in contrast to their complete absence in control siblings (Fig. 2E). These findings suggest that miRNAs are required postnatally to restrain growth of the CB and sphincter muscle, and that their requirement for epithelial adherence might occur after birth.
To delve into the molecular etiology associated with abrogated development of the CB, we focused on \textit{Dicer1loxP/loxP;\textalpha-Cre} mutants. In order to determine the extent of Dicer ablation, we characterized the expression of miR-204, which is known to be expressed in the postnatal CB (Karali et al., 2007; Ryan et al., 2006). At around P7, miR-204 is highly expressed in the pigmented and the non-pigmented layers of the CB (Fig. 3A). In the \textit{Dicer1loxP/loxP;\textalpha-Cre} mutants, miR-204 expression was absent from the non-pigmented CB layer, corresponding with \textalpha-Cre activity (Fig. 3F). This demonstrates that compensation by wild-type cells is not prevalent in the CB of \textit{Dicer1loxP/loxP;\textalpha-Cre} mutants.

On P7, the \textit{Dicer1loxP/loxP;\textalpha-Cre} CB already appeared flattened and misshapen (Fig. 3F). We therefore studied \textit{Dicer1loxP/loxP;\textalpha-Cre} at younger ages. Although CB morphogenesis occurs mostly postnatally, differential gene expression distinguishes it from the adjacent neuroretina during embryogenesis. Cx43, Gas1, Otx1 and Bmp4 are expressed in the progenitors of the CB but not of the retina (Fig. 3B-D; data not shown) (Calera et al., 2006; Lee et al., 2001; Martinez-Morales et al., 2001), and Otx1 and Bmp4 have been related to CB development (Larsen et al., 2009; Zhao et al., 2002). In \textit{Dicer1loxP/loxP;\textalpha-Cre} mutants, the expression domains of Cx43, Otx1 and Gas1 were markedly decreased compared with in control littermates (Fig. 3A). In \textit{Dicer1loxP/loxP;\textalpha-Cre} E15.5 embryos and P2 pups (Fig. 3D,E,H,I). These data suggest that Dicer1 ablation hampers the development of the CB and that miRNAs are involved in its prenatal specification or differentiation.

\textbf{The Dicer1-deficient distal retina is populated by a mixture of neuronal and non-neuronal progenitors}

We further used the \textalpha-Cre line to study miRNA function within the neuroretinal compartment of the OC. In contrast to the normal wide expression pattern of \textalpha-Cre, the expression region was diminished...
in the Dicer<sup>loxP/loxP;</sup>α-Cre mutants (see Fig. S1A,B,E,F in the supplementary material). This decrease correlated with the extensive apoptosis detected in the mutants from E14.5 onwards (Decembrini et al., 2008; Georgi and Reh, 2010) (see also Fig. S1G,H in the supplementary material).

Histological staining showed that unlike the differentiated, stratified structure of the normal retina (Fig. 4A), the mutant retina was hypocellular and atypically consisted of columnar progenitor-like cells (Fig. 4F). We characterized the phenotype of the retinal rudiment using markers for neuroretina and CB progenitors (Fig. 4). By P8, most of the retinal cells have already exited the cell cycle and downregulated the expression of progenitor markers (Fig. 4B,C). By contrast, Dicer<sup>−/−</sup> retinal cells were positive for a variety of progenitor markers, including PCNA, Ki67 (Fig. 4G,H), Pax6, Ccdn1, p27kip1 (also known as Cdkn1b) and Sox2 (not shown). Intriguingly, we observed upregulation of Ccdn3 and p57kip2 (Cdkn1c) (Fig. 4I,J), which are either absent or rarely expressed in the normal RPCs, respectively (Fig. 4D,E) (Dyer and Cepko, 2000). To delve into this phenomenon, we characterized the expression patterns of p57kip2 and Ccdn3 within the OC. On E18.5, p57kip2 was detected in selected ACs (Dyer and Cepko, 2000), whereas Ccdn3 was absent from the retina. Both markers were weakly expressed in the CB primordium on E18.5 (Fig. 4K) and were upregulated after birth (Fig. 4L). In the Dicer<sup>loxP/loxP;</sup>α-Cre retina, expression of p57kip2 and Ccdn3 was low on E18.5 and dramatically upregulated after birth (Fig. 4O,P), thus suggesting an acquisition of CB progenitor characteristics by the Dicer<sup>−/−</sup> retina. Moreover, the expression of Pax6 was detected in most of the distal retinas of mice of the Dicer<sup>loxP/loxP;</sup>α-Cre line on P1 (Fig. 4Q), similar to its high expression in the CB and iris epithelia (Fig. 4M, arrowhead). Worth mentioning is that this was not the case in either Dicer<sup>loxP/loxP;</sup>Tyrp2-Cre or Dicer<sup>loxP/loxP;</sup>Pou4f3-Cre eyes. We thus concluded that this aberrant population originates from either the non-pigmented CB or the retina itself, both of which are wild type in Dicer<sup>loxP/loxP;</sup>Tyrp2-Cre mice. Moreover, the emergence of this population is expected to occur prenatally, prior to the onset of Pou4f3-Cre activity.

Despite the expression of markers indicative of non-neuronal identity, the Dicer<sup>−/−</sup> retina also contained cells expressing the GC markers Pou4f2 (previously known as Brn3b; Fig. 4R) and beta-III-Tubulin (Tubb3; data not shown). Other retinal neurons did not differentiate in the Dicer<sup>loxP/loxP;</sup>α-Cre mutants, including photoreceptors (rodopsin and PNA; see Fig. S2B,G in the supplementary material), horizontal cells (NF165) (Fig. 4L), and amacrine cells (Fig. S2H).
in the supplementary material), bipolar cells (PKCα) (see Fig. S2C,H in the supplementary material), ACs (syntaxin 1) (see Fig. S2D,I in the supplementary material) and Müller glia (Crlbp) (see Fig. S2E,J in the supplementary material). The analysis of the neuronal subtypes formed in Dicer1-null retina corresponds with a recent study on the retinal phenotype of Dicer1loxP/loxP;α-Cre mutants (Georgi and Reh, 2010). Importantly, our results reveal that the postnatal retinal rudiment of Dicer1loxP/loxP;α-Cre mice contains a mixture of distal progenitors, as well as GCs. These findings implicate miRNA in the establishment of the border between the neuronal and non-neuronal progenitors of the OC. Furthermore, as the Dicer1loxP/loxP;Tyrp2-Cre mice did not exhibit the aberrant progenitor domain, we concluded that miRNAs in the progenitors of the iris and the pigmented CB are required for normal differentiation of the end sub-organs, whereas their function in the inner OC is in the partitioning of neuronal and non-neuronal progenitors.

We next characterized the patterning of the Dicer-null OC non-neuronal and neuronal compartments. On E15.5, neuroretinal progenitors divided extensively and expressed Sox2, Ccnd1 and Ki67. These proteins were expressed at low levels in the CB progenitors, which divide slowly at this stage (Fig. 5A-C). Additional evidence for the border between the neuronal and non-neuronal compartments was the positioning of the GCL, which on E15.5 was abutting the CB-progenitor pool (Fig. 5). In Dicer1loxP/loxP;α-Cre retinas, the GCL and the CB anlagen were spatially separated by aberrant progenitor cells, which expressed high levels of the progenitor cell markers Sox2, Ccnd1 and Ki67, thus creating a ciliary margin-like region (Fig. 5E,F,G,Q). Furthermore, an elevated number of phospho-H3+ cells was seen in this mutant region, indicative of increased mitotic activity (Fig. 5H).

A major player in the establishment of retinal identity is Chx10 (also known as Vsx2), whereas Mitf1 is a key regulator of pigmentation (Graw, 1996; Bharti et al., 2006). Mutual repression between Chx10 and Mitf1 defines the neuroretinal and pigmented progenitor domains within the OC (Horsford et al., 2005; Rowan et al., 2004). We thus monitored the expression of Chx10 and Mitf1 in Dicer1loxP/loxP;α-Cre embryos. On around E12.5 and E14.5 in control mice, Chx10 was expressed in most of the inner OC layer, abutting the marginal Mitf1-expressing cells (Fig. 5I,J). At later stages, an intermediate zone, expressing low levels of Chx10 and Mitf1, became evident between the neuronal (Chx10 high; Fig. 5K,L) and non-neuronal (Mitf1 high) domains. In the Dicer1loxP/loxP;α-Cre embryos, however, Chx10 levels were elevated distally (Fig. 5M,N) and the intermediate zone was absent (Fig. 5O,P). In agreement with the mutual-repression assumption, the expression of Mitf1 was reduced in the distal cells (Fig. 5M-O). This alteration in Chx10 and Mitf1 expression could contribute to the severe hypoplasia of the CB and to the formation of the cryptic progenitors identified in the postnatal retinas of Dicer1loxP/loxP;α-Cre mice.
Antibody staining for Ptf1a and Ap2
(A,D), Foxn4 (G,J) and Neurod1 (H,I) in the Dicer1-deficient retina (Fig. 6L). Neurod1 is a bHLH transcription factor known to be involved in AC differentiation and photoreceptor survival (Morrow et al., 1999). On E18.5, it is highly expressed along the outer aspect of the developing retina, with punctate staining within the NBL (Fig. 6G) (Morrow et al., 1999). In the Dicer1loxP/loxP;α-Cre retinas, Neurod1 expression was lost from the outer layer, with only a few cells maintaining expression in the NBL (Fig. 6J). This finding might be related to the loss of both ACs and photoreceptors in Dicer1loxP/loxP;α-Cre retinas. Taken together, this analysis corresponds with a recent study on Dicer1 deficiency in RPCs (Georgi and Reh, 2010), and is suggestive of normal specification followed by a complete failure to differentiate to the AC and photoreceptor lineages.

We then investigated the photoreceptor fate. We studied the expression of Crx1, a key factor in the formation of photoreceptors and one of their earliest markers (Fig. 6H,I) (Furukawa et al., 1997). In E15.5 Dicer1loxP/loxP;α-Cre retinas, Crx1 transcripts were correctly detected in the scleral aspect of the embryonic retina (Fig. 6K). On P2, however, Crx1 expression was lost from the Dicer1-deficient retina (Fig. 6L). Neurod1 is a bHLH transcription factor known to be involved in AC differentiation and photoreceptor survival (Morrow et al., 1999). On E18.5, it is highly expressed along the outer aspect of the developing retina, with punctate staining within the NBL (Fig. 6G) (Morrow et al., 1999). In the Dicer1loxP/loxP;α-Cre retinas, Neurod1 expression was lost from the outer layer, with only a few cells maintaining expression in the NBL (Fig. 6J). This finding might be related to the loss of both ACs and photoreceptors in Dicer1loxP/loxP;α-Cre retinas. Taken together, this analysis corresponds with a recent study on Dicer1 deficiency in RPCs (Georgi and Reh, 2010), and is suggestive of normal specification followed by a complete failure to differentiate to the AC and photoreceptor lineages.

**miRNAs are required to inhibit GC production throughout the OC**

Extensive production of GCs was observed throughout the Dicer1-deficient retinas. We therefore examined the dynamics of GC formation in Dicer1loxP/loxP;α-Cre mutants. GCs are the earliest retinal cell type, with the last cell born on around E14.5 (Young, 1985). The GC precursors migrate to and populate the GCL (Fig. 7A,L). In Dicer1loxP/loxP;α-Cre embryos, however, a considerable number of Pou42+ cells populated the NBL on E15.5 and E18.5 (Fig. 7E,P). Double staining with the cell-cycle marker Ki67 demonstrated an 84% elevation in the number of Pou42+ cells in the NBL on E18.5 (s.d. = 152%, n = 5, P < 0.001) in comparison to the control retinas (s.d. = 28.4%, n = 3). Accordingly, the GCL was 46% thicker in the mutants (s.d. = 14%, n = 5, P < 0.001) than in the controls (s.d. = 5%, n = 3).

To confirm the identity of these cells as GCs and to rule out a specific upregulation of Pou42, we performed the same analysis with Isl1. Normally, Isl1 is expressed in a distinct, yet overlapping population of GCs, as well as in differentiating ACs and bipolar cells (Mu et al., 2008). On E18.5, Isl1+ cells are mostly confined to the GCL and to the presumptive INL (Fig. 7B). In the mutants, we observed a higher proportion of Isl1+ cells in the NBL (63.1%, s.d. = 12.6%, n = 4, P < 0.005) than in the NBL of controls (s.d. = 31%, n = 2), supporting the notion that the mislocated Pou42+ cells are indeed GCs (Fig. 5F).

The presence of GCs in the NBL could be interpreted as an uncoupling of proliferation and differentiation. To test this, we performed double staining with cell-cycle markers (i.e. Ki67 and PCNA) and Pou42 at various developmental stages (E15.5, E17.5, P1 and P8). We found that soon after the birth of the GCs, there is a short temporal overlap between the decay of cell-cycle markers and the onset of Pou42 expression. In control retinas, this phenomenon was seen until E15.5 but, in the mutants, it extended to perinatal days (not shown). This finding implied that the uncoupling of proliferation and differentiation is not impeded in the absence of Dicer1, but that the timeframe of the birth of GCs might be prolonged. We therefore performed a pulse-chase experiment in which embryos were injected with BrdU on E17.5 and harvested on either P1 or P8. In P1 control embryos, BrdU+ cells were detected in the presumptive INL and ONL (Fig. 7C). On P8, BrdU+ cells populated the INL and ONL, with a few BrdU+ cells in the GCL, marking displaced ACs (Fig. 7D). No colocalization...
of BrdU and Pou4f2 was observed in the P1 or P8 control mice. In Dicer1<sup>flaxPloxP;α-Cre</sup> retinas, however, BrdU+;Pou4f2+ cells were prevalent on P1 (Fig. 7G), but not on P8 (Fig. 7H), suggesting that some GCs were anachronistically born on E17.5, but failed to survive until P8.

To explore molecular alterations related to the aforedescribed phenomena, we examined the notch and hedgehog (Hh) pathways, which are implicated in neurogenesis and GC production (Jadhav et al., 2006a; Jadhav et al., 2006b; Riesenber et al., 2009; Yaron et al., 2006; Furimsky and Wallace, 2006; Sakagami et al., 2009; Wang et al., 2005). Notch1 and its downstream target Hes5 are normally expressed in the neuronal, but not in the non-neuronal, progenitors of the OC (Fig. 7I,J) (Bao and Cepko, 1997; Kageyama and Ohtsuka, 1999; Yaron et al., 2006). In Dicer1<sup>flaxPloxP;α-Cre</sup> retinas, however, marked downregulation of Notch1 and Hes5 transcript was detected on around E17.5 (Fig. 7M,N). Examination of Dicer1<sup>flaxPloxP;α-Cre</sup> eyes demonstrated that the ganglion phenotype is already evident on E15.5 (Fig. 7L,P). Nonetheless, Notch1 expression was normal at this stage, whereas Hes5 appeared elevated when compared with littermate controls (Fig. 7Q,R,U,V). This might reflect a diminution of precursors rather than a direct alteration in Hes5 expression. This analysis therefore did not support involvement of the Notch pathway in mediating the elevation in GC production.
To monitor Hh activity in Dicer1loxP/loxP;α-Cre retinas, we analyzed the expression of Gli1, a transcriptional mediator and downstream target of the Hh pathway (Parisi and Lin, 1998). In E17.5 control retinas, Gli1 displayed dual expression: high in the NBL and low in the GCL (Fig. 7K) (Dakubo et al., 2008). In Dicer1loxP/loxP;α-Cre retinas, however, only a low level of Gli1 transcript was detected (Fig. 7O). Decreased expression was also evident on E15.5, a finding that coincides temporally with the formation of extra GCs in the mutants (Fig. 7S,W). To verify the inhibition of Hh signaling at this stage, we looked at the expression of Patched1 (Ptc1), a co-receptor of Smo and a downstream target of the pathway, and found a similar downregulation on E15.5 (Fig. 7T,X). Considering the delay in miRNAs degradation described in Dicer1 conditional lens mutation (Li and Piatigorsky, 2009) and that the changes in GC distribution were initiated after E12.5 (data not shown), we conclude that the inhibition of Hh signaling is a relatively early result of Dicer1 ablation and correlates with the misregulation of GC production.

**DISCUSSION**

This study describes the roles of Dicer in the developing retina and anterior structures of the eye cup. Dicer1 was found to be required for the partitioning of neuronal and non-neuronal progenitors in the distal OC, for differentiation of most retinal cell types and for regulation of timing of production and number of GCs. The changes caused by ablation of Dicer1 were associated with perturbations in the expression of Notch- and Hh-signaling-responsive genes. Furthermore, the study revealed a requirement for miRNAs in the adhesion properties of epithelial cells of the iris and CB, and in restricting growth of the CB and iris cell types at postnatal stages.

**Tissue-specific roles for miRNA in iris development**

The iris forms from two embryonic origins: the OC, which gives rise to the muscles and the epithelia; and the pericocular mesenchyme, which contributes to the stroma (Davis-Silberman and Ashery-Padan, 2008). Here, we identified miRNA functions within the different iridial tissues during embryonic and postnatal development. The phenotype of Dicer1loxP/loxP;α-Cre mutants suggests that miRNAs in the inner OC are dispensable for the formation of the IPE and muscles, but act at later stages in the adherence of the epithelial layers (Fig. 1M). Interestingly, detachment of epithelial cells following Dicer1 loss has been described in lung epithelium and kidney cell types (Harris et al., 2006; Harvey et al., 2008; Ho et al., 2008). This, together with our findings, implicates a general role for miRNA in the regulation of cell-adherence-related molecules.

Despite the overlapping expression of the α-Cre and Typr2-Cre transgenes within the iris, the latter Cre line was uniquely active within the iris stroma. The severity of the iridal phenotype of Dicer1loxP/loxP;Typr2-Cre mutants indicates the importance of Dicer1 activity in the stroma for morphogenesis of the adjacent iris layers. The formation of the stroma includes migration of mesenchymal cells into the anterior eye, adherence to the iris scaffold, proliferation and differentiation (Cvekl and Tamm, 2004; Smith, 2002). All of these biological processes can potentially involve miRNA regulation of gene expression.

Finally, the use of the late-acting Pou4f3-Cre transgene enabled us to examine the role of miRNA in the late differentiation of the iris, precluding the early patterning defects of the Dicer1loxP/loxP;α-Cre. Surprisingly, late ablation of Dicer1 in the anterior structures led to a persistence of cycling cells in the mature CB and to a marked overgrowth of the sphincter muscle (Fig. 2H,J). Owing to the mosaic pattern of recombination mediated by the Pou4f3-Cre transgene, the phenotypes might at least partly reflect a non-cell-autonomous response of Dicer+ cells to their mutant neighbors. This finding suggests that miRNA might act to restrain the size of the vision accessorial structures, a function needed for the coordinated growth of the eye.

**CB formation requires Dicer1 function**

The first event occurring during morphogenesis of the murine ciliary processes is a proliferative surge of the pigmented epithelium (Napier and Kidson, 2005). After a slight delay, the non-pigmented epithelium responds by proliferating and extending inward towards the lens. This coordinated development entails close contact of the two epithelial layers. Indeed, targeting the gap junction or cell-adhesion molecules Cx43 and nectin-1/nectin-3 (also known as Pvr1/Pvr13), localized at the junctions between the pigmented and non-pigmented layers, disrupts formation of the CB (Calera et al., 2006; Inagaki et al., 2005). Here, we show that exclusive ablation of Dicer1 from either the non-pigmented (Dicer1loxP/loxP;α-Cre) or pigmented (Dicer1loxP/loxP;Typr2-Cre) layers seriously hampers the development of both epitelia, emphasizing the vital and reciprocal role of the two layers in the formation of each other (Fig. 1I,J,N,O). Furthermore, targeting Dicer1 activity on postnatal days using the Pou4f3-Cre line demonstrated that this role is mainly played at the progenitor stage, whereas at postnatal stages miRNAs act primarily to restrain CB growth (Fig. 2L,J). To date, the only miRNA localized to the CB epithelium has been miR-204 (Ryan et al., 2006) (Fig. 3A). A recent study demonstrated reduced expression of miR-204 in a NCI60 tumor cell line panel, suggesting an inhibitory function on cell proliferation (Wang et al., 2010). In the same study, miR-204 was implicated in maintenance of the epithelial barrier in pigmented epithelium. The loss of miR-204 might mediate the increase in cell divisions, as well as the epithelial detachments, seen in Dicer1loxP/loxP;Pou4f3-Cre and Dicer1loxP/loxP;α-Cre mice.

**miRNAs are required for late patterning of the distal OC**

Several pieces of evidence, mostly from amphibians and birds, suggest that partitioning of the neuroretina and CB domains is labile up to at least the late stages of development (Mitashov, 2001; Fischer and Reh, 2001; Mosheri et al., 2004; Spence et al., 2004). In mammals, it is not clear when the borders between the neuronal and non-neuronal compartments of the OC are irreversibly defined. An indication of two distinct progenitor pools is evident in the mouse at around mid-gestation (Davis et al., 2008). Cell-cycle-related factors are differentially expressed by the progenitors of both the CB and the retina, with a decreasing gradient from retina to CB (Fig. 5A-C) (Fischer and Reh, 2000; Liu et al., 2007). Our data demonstrate that cells located at the distal tip of Dicer1loxP/loxP;α-Cre embryos ectopically express high levels of progenitor markers (Fig. 5E-G). The elevated expression of Chx10 in these cells (Fig. 5O,P) and the decreased size of the CB domain (Fig. 3G,H) suggest that this ectopic upregulation results from an undermining of the non-neuronal identity of the distal cells. Close to birth, however, this cryptic population upregulates the expression of CB-progenitor genes, with a timing that matches their normal onset within the CB (e.g. Ccnd3, p57 and Pax6; Fig. 40-Q). At the same time, the expression of some neuronal factors [Neurod1 and Crx1 (Fig. 6J,K,L); Notch1 and...
Hes5 [Fig. 7M,N] decreases. It is possible that these molecular alterations merely result from ablation of certain miRNAs; however, when considering the embryonic distal origin of these cells, together with the upregulation of Chx10, it is tempting to speculate that the mutant cells possess a mixed CB/retinal identity. This interpretation proposes a novel role for miRNAs in the maintenance of the neuronal/non-neuronal border of the OC. Notably, despite the broad area of α-Cre expression (see Fig. S1A,E in the supplementary material), only cells located at the retina-CB junction acquired the cryptic phenotype. This is further supported by the fact that ablation of Dicer1 by Chx10-Cre, which is mostly active in patches of the proximal retina, does not result in the described changes (Damiani et al., 2008).

miRNA involvement in retinal differentiation

Several studies have correlated miRNAs and differentiation potential. In *Caenorhabditis elegans*, post-transcriptional regulation of the heterochronic gene *lin-14* by lin-4 mediates temporal pattern formation (Wightman et al., 1993), and the 21-nucleotide let-7 RNA regulates developmental timing (Reinhart et al., 2000). Knockout of either Dicer1 or Dgcr8, an RNA-binding protein involved in miRNA processing, results in differentiation failure of embryonic stem (ES) cells (Kanellopoulou et al., 2005; Wang et al., 2007). Finally, inactivation of Dicer1 in the *Xenopus* retina increases the number of cycling cells and causes a delay in cell birth (Decembrini et al., 2008). The latter findings correspond with the persistence of progenitors within the postnatal Dicer1<sup>loxPloxP</sup>, α-Cre retina (this study) (Georgi and Reh, 2010) and with the non-pigmented CB in Dicer1<sup>loxPloxP</sup>, Pou4f3-Cre mice. It is worth mentioning that there are studies (Ahmad et al., 2000; Tropepe et al., 2000) suggesting that there is a reservoir of retinal stem cells within the CB. Those cells, however, were derived from the pigmented layer of the CB rather than the non-pigmented one and thus, might not be related to the cryptic progenitors identified here following Dicer1 inactivation.

miRNAs might safeguard against signals that could otherwise simultaneously activate alternative differentiation programs within the same cell lineage (Hornshøj and Shomron, 2006); thus, the observed role of miRNAs in retina/CB partitioning could reflect a more general miRNA-based mechanism underlying the differentiation of progenitors, along with controlled diminution of the self-renewal program. Indeed, this idea is compatible with previous studies addressing the roles of miRNAs in a variety of developmental contexts. For example, miR-203 promotes epidermal differentiation by restricting proliferative potential and inducing cell-cycle exit (Yi et al., 2008). Similarly, miR-430 represses maternally contributed mRNA, thus promoting transition to the zygotic program (Giraldez et al., 2006). Finally, mutually exclusive cross-regulation of the miR-290-295 cluster and let-7 is essential for mouse developmental program (Nat. Genet. 35, 215-217).

The regulation of proliferation versus differentiation by miRNA might be intertwined with known regulatory mechanisms, such as Notch and Hh signaling. Recent studies on the functions of Notch-signaling genes support a role for this pathway in maintaining the progenitor pool and in inhibiting GC and photoreceptor lineages (Jadhav et al., 2006b; Yaron et al., 2006; Riesenber et al., 2009). Dicer loss resulted in a complex, stage-dependent perturbation of the Notch-pathway genes: Hes5 expression was elevated on E15.5 but lost on E17.5. Moreover, whereas Notch activity normally inhibits the expression of most proneural genes (Jadhav et al., 2006b; Yaron et al., 2006), in the Dicer1-deficient retina, Neurod1 expression was lost and Math3 was not altered on E17.5, despite the loss of Hes5 and Notch1 at this stage (Fig. 6K; N.D. and R.A.-P., unpublished observations). These findings reflect the complex effects of miRNAs on the Notch components and suggest that, either directly or indirectly, miRNAs play a role in regulating the proneural genes, in particular Neurod1.

In mammals, the conditional inactivation of Hh signaling in RPCs leads to a bias toward a retinal ganglion fate, resulting in an increase in the number of GCs (Sakagami et al., 2009; Wang et al., 2001). Furthermore, it has been shown that retroviral-mediated overexpression of Shh results in reduced GC proportions in vivo and in vitro (Zhang and Yang, 2001). These findings support the possibility that downregulation of the Shh targets Gli1 and Ptc1 might contribute to the GC phenotype of Dicer<sup>−−</sup> retina. The challenge for future studies will be to decipher the gene networks regulated by specific miRNA families that mediate their pleiotropic functions in retinogenesis and development of the sub-organs of the eye.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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