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RESEARCH ARTICLE

MicroRNAs are essential for differentiation of the retinal pigmented epithelium and maturation of adjacent photoreceptors

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ABSTRACT

Dysfunction of the retinal pigmented epithelium (RPE) results in degeneration of photoreceptors and vision loss and is correlated with common blinding disorders in humans. Although many protein-coding genes are known to be expressed in RPE and are important for its development and maintenance, virtually nothing is known about the *in vivo* roles of non-coding transcripts. The expression patterns of microRNAs (miRNAs) have been analyzed in a variety of ocular tissues, and a few were implicated to play role in RPE based on studies in cell lines. Here, through RPE-specific conditional mutagenesis of *Dicer1* or *Dgcr8* in mice, the importance of miRNAs for RPE differentiation was uncovered. miRNAs were found to be dispensable for maintaining RPE fate and survival, and yet they are essential for the acquisition of important RPE properties such as the expression of genes involved in the visual cycle pathway, pigmentation and cell adhesion. Importantly, miRNAs of the RPE are required for maturation of adjacent photoreceptors, specifically for the morphogenesis of the outer segments. The alterations in the miRNA and mRNA profiles in the Dicer1-deficient RPE point to a key role of miR-204 in regulation of the RPE differentiation program *in vivo* and uncover the importance of additional novel RPE miRNAs. This study reveals the combined regulatory activity of miRNAs that is required for RPE differentiation and for the development of the adjacent neuroretina.

KEY WORDS: RPE, Dicer1, Dgcr8, MicroRNA, Eye development, Photoreceptor, Mouse

INTRODUCTION

Normal vision depends on the retinal pigmented epithelium (RPE), which comprises pigmented epithelial cells that reside between the blood vessels of the choriocapillaris and the light-sensitive outer segments of the photoreceptors. Although not an intrinsic component of the visual signaling pathway, the RPE is a highly metabolic cell layer that is vital to the health, survival and function of the adjacent retinal photoreceptor cells.

RPE cells are physically and functionally associated with the photoreceptor cells. The apical microvilli of the RPE engulf the outer segment of the photoreceptors, while the basal layer is associated with the Bruch's membrane underlying the choriocapillaris (Burke, 2008). As a layer of pigmented epithelial cells, the RPE is essential for absorbing stray light that would otherwise degrade the visual image and cause photoreceptor damage (Strauss, 2005). Importantly, the RPE takes an active part in the so-called visual cycle, as it expresses the enzymes required for re-isomerization of all-*trans* retinal to 11-*cis* retinal and for its transport back to photoreceptors to regenerate visual pigments and complete the visual cycle [e.g. LRAT, RPE65, CRALBP (RLBP1), IRBP (RBP3)] (Batten et al., 2004; Jin et al., 2005; Travis et al., 2007). All-*trans* retinol is also supplied to the RPE by the choroidal vasculature, entering the RPE in a receptor-mediated process involving recognition of a serum retinol-binding protein/transthyretin (RBP-TTR) complex (Thompson and Gal, 2003).

Vertebrate rod and cone photoreceptors are highly specialized, polarized neurons that are divided into several morphologically and functionally distinct compartments: a synaptic terminal, an outer segment, an inner segment, and a cilium connecting the outer and inner segments (Insinna and Besharse, 2008). Outer segments (OSs) are formed initially from the primary cilia in photoreceptor precursors. Like other cilia, the photoreceptor cilia contain an axoneme, which begins at the basal bodies and passes through a transition zone, also called the connecting cilia, and into the OS.

The later stages of RPE and photoreceptor development occur during postnatal life, as photoreceptor cells begin to mature morphologically and RPE cells are activated to undergo the last stages of differentiation (Marmorstein et al., 1998). As the photoreceptor cells form connecting cilia and extend OSs, RPE cells respond by extending microvilli into the subretinal space (Marmorstein et al., 1998). The microvilli begin to surround the growing OS of photoreceptors and, by the end of differentiation, the RPE has developed two types of microvilli: long microvilli that maximize the apical surface for epithelial transport, and shorter microvilli that form photoreceptor sheaths for phagocytosis of the photoreceptor OS. Accompanying the onset of microvilli growth at the apical membrane is the formation of deep basal infoldings in the basolateral membrane. The interaction between the RPE and photoreceptor OS appears to be mediated by factors secreted from the RPE. *In vitro* studies have shown that medium from RPE cultures is sufficient to promote photoreceptor differentiation and survival (Gaur et al., 1992; Spoerri et al., 1988) and pigmented epithelium-derived factor (PEDF; also known as SERPINF1) supports photoreceptor development (Jablonski et al., 2000).

Because photoreceptor cells depend heavily on the RPE for survival, pathologies associated with the RPE can lead to photoreceptor cell degeneration and impaired vision. In particular, defects in RPE cells can result in age-related macular degeneration (AMD), which is a common cause of impaired vision in humans, as well as the congenital blindness of retinitis pigmentosa (RP) (Ambati et al., 2003; Wright et al., 2010). Furthermore, genetic

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ablation of the RPE or disruption of RPE specification genes results in microphthalmia, which is associated with transdifferentiation of RPE cells to neural retinal cells (Bharti et al., 2006; Martinez-Morales et al., 2004; Zhao et al., 1997). Understating the underlying mechanisms by which RPE cell fate is normally acquired and maintained and the interactions between the RPE and adjacent retina is pivotal for understanding the etiology of RPE dysfunctions.

MicroRNAs (miRNAs) are small non-coding RNAs that were initially discovered in *C. elegans* and emerged as evolutionarily conserved, post-transcriptional repressors that bind to the 3′-UTR of mRNA targets (Lee et al., 2001; Reinhart et al., 2000). miRNAs control gene expression by regulating the stability and/or translation efficiency of different mRNAs. A single miRNA may directly alter the expression of hundreds of proteins to a mild extent, and indirectly affect the expression of thousands of proteins (Baek et al., 2008; Selbach et al., 2008). Although the effect on the level of each protein is, in most cases, mild, the accumulation of effects may induce a significant phenotype. Thus, miRNAs are crucial for development, with specific miRNAs linked to the development of specific organs (Maatouk and Harfe, 2006).

The miRNA is transcribed as a primary miRNA (pri-miRNA) by RNA polymerase II and is then cleaved by the nuclear microprocessor complex formed by the RNase III enzyme Drosha and the DGCR8 protein (Winter et al., 2009). The resulting precursor hairpin, the pre-miRNA, is exported to the cytoplasm where it is cleaved by the RNase III nuclease Dicer1 to generate the ∼22 nucleotide miRNA duplex that is incorporated into the RNA-induced silencing complex (RISC) (Grishok et al., 2001). In the absence of Dicer1, little or no miRNAs are produced (Schier and Giraldez, 2006). *Dicer1* mutant mice die on E7.5 (Bernstein et al., 2003). Conditional inactivation of *Dicer1* and *Dgcr8* has been performed in various organs, exposing the roles of miRNAs in various developmental processes (Maatouk and Harfe, 2006; Yi et al., 2009).

The expression patterns of miRNAs have been analyzed in a variety of ocular tissues and several have been identified in mouse and human RPE cell lines (Karali et al., 2010; Wang et al., 2010). To date, functional studies on a few miRNAs have been conducted in primary cell cultures, revealing their roles in the regulation of angiogenesis of the choriocapillaris, in prevention of oxidative damage, and in maintaining barrier functions and epithelial physiology of cultured RPE (Haque et al., 2012; Lin et al., 2011; Wang et al., 2010). Moreover, miRNAs are associated with endoplasmic reticulum stress, which is a hallmark of AMD and results in altered expression of tight-junction molecules and apoptosis genes (Byrd et al., 2012; Chitnis et al., 2012; Yoshikawa et al., 2011). These findings led to the hypothesis that miRNAs are involved in processes that are crucial for the normal physiology of the RPE and of the adjacent tissues, namely the choroid and retina. Conditional mutation of *Dicer1* in the adult RPE revealed its role in the degradation of transposon RNA (Kaneko et al., 2011). This activity, which is essential for cell survival, precluded any attempt to expose miRNA functions *in vivo* (Kaneko et al., 2011). The main goal of this study was to determine the *in vivo* functions of miRNAs during normal development of the RPE and their involvement in diseases of the RPE and retina.

RESULTS

Conditional ablation of *Dicer1* from RPE cells as a global indicator of miRNA activity

To explore the role of miRNAs in RPE development and function, we established a conditional deletion of *Dicer1* in the developing RPE using a *Dct-Cre* transgene and a *Dicer1*^{loxP} allele (Davis et al., 2009, 2011; Harfe et al., 2005). The recombination activity of *Dct-Cre* is initiated early at the optic vesicle stage (E9.5) on its dorsal side and, later on, at the optic cup (OC) stage recombinant cells are distributed over the entire RPE (Davis et al., 2009; Zhao and Overbeek, 1999). We extended the analysis of *Dct-Cre* activity to P9, when retinal lineages differentiate, and we used the *Z/EG* reporter, which enables Cre activity to be monitored at cellular resolution by detection of GFP reporter expression (Novak et al., 2000). The GFP reporter was detected throughout the *Z/EG; Dct-Cre* RPE, while in the neuroretina we detected sparse labeling in 7±1% (±s.d., *N*=4) of photoreceptors, which were located in the peripheral but not central neuroretina (supplementary material Fig. S1). This analysis substantiates robust recombination, which is mostly restricted to the pigmented ocular cells. *Dct-Cre* mice were crossed with the *Dicer1*$^{loxP/loxP}$ transgenic line to create heterozygous *Dicer1*$^{loxP/+}$*;Dct-Cre* mice. These mice were phenotypically normal in all respects that we examined. Throughout this study the *Dicer1*$^{loxP/loxP}$*;Dct-Cre* were compared with *Dicer1*$^{loxP/loxP}$ control littermates, which are indistinguishable from wild-type mice.

Corresponding with the pattern of *Dct-Cre* activity, we observed significant reduction of *Dicer1* transcripts from the RPE, but not the neuroretina, at postnatal stages by QPCR using primers that detect the region that is deleted by Cre from the *Dicer1*^{loxP} allele (supplementary material Fig. S1C, Table S4) (Harfe et al., 2005). To monitor the loss of Dicer1 activity during embryogenesis we characterized the expression of miR-211 and miR-204, which are nested within the genes *Trpm1* and *Trpm3*, respectively, by *in situ* hybridization (ISH). These miRNAs were chosen because they are known to be highly expressed in the RPE throughout development and adult life (Shaham et al., 2013; Wang et al., 2010). Furthermore, miR-204 has been implicated in the inhibition of factors involved in epithelial-to-mesenchymal transition (EMT) and has been shown to be required for epithelial integrity as well as for maintaining primary cultures of human RPE in a non-proliferative state (Adijanto et al., 2012). The reduction of both miR-204 and miR-211 was clearly evident from E14.5 in the *Dicer1*$^{loxP/loxP}$*;Dct-Cre* RPE (Fig. 1A-D), whereas *Trpm3* expression, which contains miR-204, was maintained (Fig. 1E,F). The results showed a specific embryonic reduction of miRNAs in the *Dicer1* mutant RPE. Notably, *Trpm3* distribution and Hematoxylin and Eosin (H&E) staining showed that the mutant RPE sustains its monolayer morphology throughout embryogenesis, although a slight reduction in cell size was noted in the *Dicer1* mutant cells (Fig. 1G-H′ and Fig. 2).

We next performed a more detailed phenotypic analysis on postnatal eyes through P11 (see below). The analysis was restricted to P11 because subsequently there was loss of the RPE in *Dicer1* mutants. This could be a secondary consequence of the altered development of other ocular tissues that was observed in the *Dicer1* mutant eyes, including the lack of an anterior segment and vitreous and the abnormal development of photoreceptors (Davis et al., 2009) (see Figs 5 and 6), as well as a requirement of Dicer1 for degradation of transposon RNA, which is associated with RPE death in the adult eye (Kaneko et al., 2011).

Dicer1 is dispensable for RPE fate and gross morphology but is required for normal cell size and RPE function

We next aimed to determine whether RPE fate is altered due to Dicer1 loss. Histological staining (Fig. 2A,B), revealed that the single-layer epithelial morphology was maintained. Furthermore, we detected the expression of transcription factors that participate

Development (2015) 142, 2487-2498 doi:10.1242/dev.121533

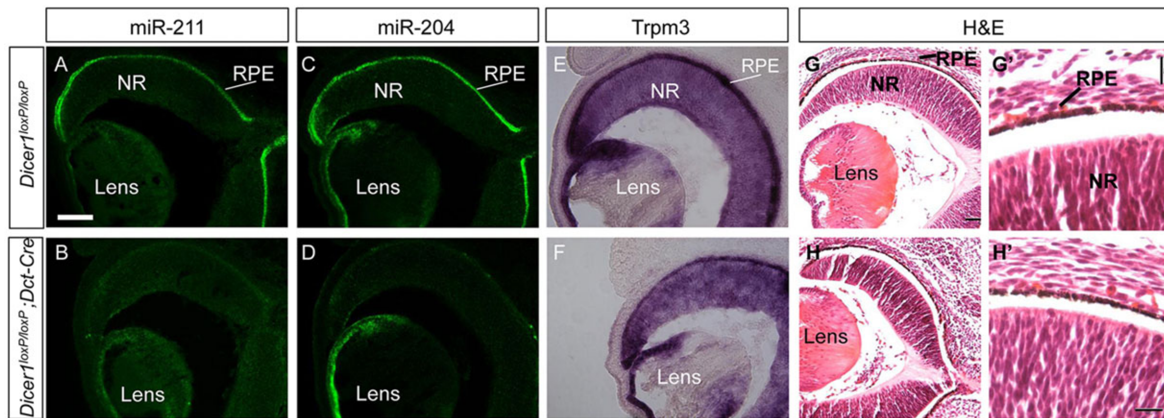

Fig. 1. miR-211 and miR-204 are lost in the *Dicer1*$^{loxP/loxP}$*;Dct-Cre* embryonic RPE. miRNA expression analysis conducted on control (top row) and *Dicer1*$^{loxP/loxP}$*;Dct-Cre* (bottom row) mouse eyes at E14.5. (A-D) Expression of miR-211 and miR-204, as detected using ISH, was reduced in the mutant RPE (B,D, green) as compared with control RPE (A,C, green). (E,F) Expression of *Trpm3*, the host gene of miR-204, was maintained in the Dicer1-deficient RPE (F) as compared with control RPE (E). (G-H′) Hematoxylin and Eosin (H&E) staining at low (G,H) and higher magnification (G′,H′). NR, neuroretina; RPE, retinal pigmented epithelium. Scale bars: 100 µm in A-H; 20 µm in G′,H′.

in RPE specification and maintenance, namely *Otx2* and *Mitf* as monitored by QPCR (Fig. 2C) and Sox9 detected by antibody labeling (Fig. 2D-E′) (Martinez-Morales et al., 2004; Masuda and Esumi, 2010; Masuda et al., 2014; Nguyen and Arnheiter, 2000). In addition, we did not detect upregulation of Chx10 (Vsx2 – Mouse Genome Informatics), an early expressed retinal transcription factor, indicating that the RPE was not transdifferentiating into neural retinal cells in the Dicer-deficient RPE (Fig. 2F-G′) (Horsford et al., 2005).

Although the gross morphology and identity of the RPE were maintained, several alterations in the RPE phenotype were observed. Normally, the RPE cells are pigmented and exhibit regular hexagonal morphology as detected in flat-mounted RPE stained with phalloidin (P5, Fig. 3A-B′) (Chrenek et al., 2012). Moreover, the RPE cells are polarized, with apical microvilli and basal infoldings that can be imaged by TEM (Burke, 2008) (P11, Fig. 3C). The cellular polarity is also evident by the localization of the scaffold protein phospho-ezrin (p-Ezrin) to the apical microvilli (Fig. 3D,D′) (Bonilha et al., 1999; Viswanatha et al., 2014). By contrast, in the *Dicer1*$^{loxP/loxP}$*;Dct-Cre* mice the RPE cells were depigmented and smaller than normal, which resulted in a twofold increase in cell density (Fig. 3E-F′,I). However the Dicer1-deficient cells did preserve their typical hexagonal morphology, based on quantitative analysis of the number of neighbors (Fig. 3J) (Chrenek et al., 2012). At P11, the apical microvilli appeared to be reduced based on TEM analysis (Fig. 3G) and the distribution of p-Ezrin in the *Dicer1* mutant was aberrant (Fig. 3H,H′). Finally, vacuoles were detected in the Dicer1-deficient cells at P11, a defect that is commonly associated with mutations affecting the RPE (Fig. 3G) (Ameen and Salas, 2000; Bonilha et al., 2006; Saint-Geniez et al., 2009). Taken together, although Dicer1 seems to be dispensable for the single-layer hexagonal morphology of the RPE and for the apical accumulation of p-Ezrin, it is required for proper cell density and normal apical microvilli morphology.

Normal vision depends on continuous re-isomerization of all-*trans* retinal to 11-*cis* retinal in photoreceptor and RPE cells. At P5, in both *Dicer1* and *Dgcr8* mutant RPE, the expression of key enzymes and related proteins in this process was severely reduced by several fold, as shown for *Rpe65* [−25.78 fold change (FC), *P*=0.02], *Lrat* (−16.75 FC, *P*=0.009) and *Ttr* (−8.29 FC, *P*=0.007) (Fig. 4A). Nor did we detect Rpe65 and Cralbp protein expression in the Dicer1-deficient RPE, in contrast to control littermates at P11 (Fig. 4B-E).

These results indicated the important role of Dicer1, Dgcr8 and, inferring from this, of miRNAs in the proper expression of visual cycle components. Overall, these findings suggested that miRNAs are not essential for maintaining the specification of RPE cells but are essential for the execution of distinct differentiation programs, including pigmentation and the expression of visual cycle components.

Failure of photoreceptor OS formation and retinal degeneration in Dicer1-deficient RPE

Although most photoreceptors were of normal genotype, based on the pattern of Dct-Cre activity and on QPCR for *Dicer1* in the neuroretina at P9 (supplementary material Fig. S1), there was a complete absence of photoreceptor OSs throughout the entire retina (Fig. 5A-D). We examined the surface of the outer retina by SEM. In the *Dicer1*$^{loxP/loxP}$*;Dct-Cre* eye, the surface of the retina consisted of inner segments with bare connecting cilia, whereas in the *Dicer1*$^{loxP/loxP}$ retina these structures were largely obscured by newly formed OSs that extend from the connecting cilia and cover the surface (Fig. 5E,F). These results demonstrate an unanticipated non-cell-autonomous requirement of miRNAs in the RPE for proper OS morphogenesis in the adjacent photoreceptor layer of the retina.

OSs are initially formed from the connecting cilium (Kennedy and Malicki, 2009). To determine whether Dicer1-deficient mice undergo normal ciliogenesis we examined photoreceptor cilia at P5, when OSs have yet to develop but connecting cilia are clearly visible (supplementary material Fig. S2A,A′). In mutants, the connecting cilia also projected normally from the inner segments (supplementary material Fig. S2B,B′). Together, these results indicated that the absence of miRNAs in the RPE has no effect on the normal onset of ciliogenesis. However, RPE miRNAs are essential for proper OS morphogenesis.

Aberrant accumulation of OS proteins in the outer nuclear layer in Dicer1- and Dgcr8-deficient RPE

Rhodopsin, which is responsible for initiating the first steps in light-dependent signal transduction, was localized specifically to the OS by P11 in control mice (Fig. 6A). Although *Dicer1*$^{loxP/loxP}$*;Dct-Cre* retinas also exhibited strong rhodopsin expression, it was aberrantly localized within the cell bodies of the outer nuclear layer and within

Development (2015) 142, 2487-2498 doi:10.1242/dev.121533

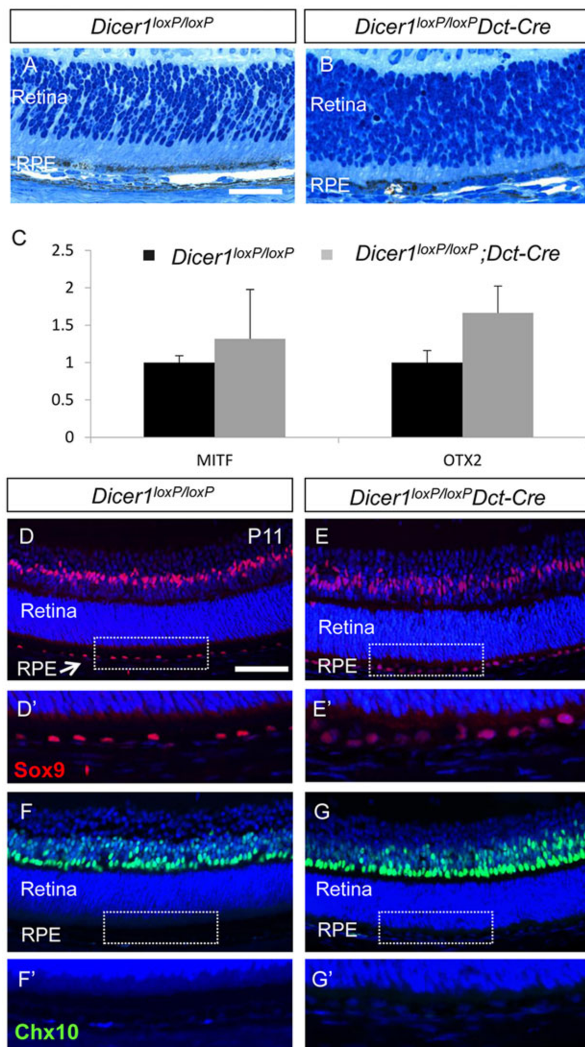

Fig. 2. RPE gross morphology and fate are maintained in Dicer1-deficient RPE. (A,B) Semi-thin sections of P11 eye stained with Toluidine Blue showing the single-layer structure of the RPE in control (A) and *Dicer1*$^{loxP/loxP}$*;Dct-Cre* (B) eyes. (C) QPCR for monitoring expression of *Mitf* and *Otx2*. Error bars indicate s.d. (D-G′) Antibody labeling on sections of *Dicer1*$^{loxP/loxP}$ (D,D′,F,F′) and *Dicer1*$^{loxP/loxP}$*;Dct-Cre* (E,E′,G,G′) retina for detection of Sox9 (D-E′) or Chx10 (F-G′). Boxed regions are shown at higher magnification beneath. Scale bars: 100 µm.

the inner segments of the photoreceptors (Fig. 6B; supplementary material Fig. S3). A similar distribution of rhodopsin was found when we deleted *Dgcr8* in the RPE using *Dgcr8*$^{loxP/loxP}$*;Dct-Cre* (Fig. 6C; supplementary material Fig. S3), which further supports that this phenotype is due to loss of miRNAs in the RPE, rather than to any miRNA-independent activities of Dicer1.

Accumulation of rhodopsin in the inner segments and photoreceptor soma leads to initiation of apoptotic cell death (Fliegauf et al., 2007). Indeed, apoptosis within the photoreceptor layer was detected in the Dicer1-deficient RPE, as assessed by activated caspase 3 expression (supplementary material Fig. S3). Moreover, histological analysis of retinas from *Dicer1*$^{loxP/loxP}$*;Dct-Cre* mice showed rapid loss of the photoreceptor layer, which by P19 was severely diminished (supplementary material Fig. S3). These data suggested that miRNAs of the RPE are required for the normal development of the OS and eventually for the survival of the photoreceptors.

Reduced expression of specific miRNAs in Dicer1-deficient RPE

To identify the miRNAs involved in later stages of RPE development, we profiled miRNAs from micro-dissected RPE of *Dicer1*$^{loxP/loxP}$ and *Dicer1*$^{loxP/loxP}$*;Dct-Cre* mice at P5 using Affymetrix GeneChip miRNA arrays (2.0) (GEO accession GSE69883). Differences were already observed in Dicer1-deficient RPE at P5. Twenty miRNAs were significantly downregulated (FC<−1.5, P<0.05), of which six were with a false discovery rate below 10% (FDR<0.1; Table 1; supplementary material Table S1). We detected reduced expression of miR-204 (−11.16 FC, P=1.45×10^{-5}), which is known to be highly enriched in the RPE (Karali et al., 2007). miR-344 was downregulated dramatically (−19.39 FC, P=1.94×10^{-6}); however, we did not follow its expression further as this miRNA is specific to rodents without known homologs in other species (Landgraf et al., 2007). Other reported RPE signature miRNAs, such as miR-222 (−1.86 FC, P<3×10^{-4}) and miR-221 (−1.62 FC, P<0.001), were also reduced in mutant RPE (Hou et al., 2013; Wang et al., 2010). We also detected a reduction of miRNAs not previously shown to be expressed in RPE, including miR-20b and miR-106a, which share target sequence, as well as miR-708.

To validate the microarray results, we performed QPCR analysis of several miRNAs that were significantly downregulated in the microarray profiling and are present in humans and mice. Consistent with the microarray data, miR-222 showed significant downregulation in the mutant RPE (Fig. 7; −2.64 FC, P<0.04). miR-20b and miR-106, neither of which had previously been shown to be expressed in the RPE, were also significantly reduced in expression (Fig. 7; −6.25 FC, P<2×10^{-4} and −5.6 FC, P<4.5×10^{-4}, respectively). We included an examination of miR-155, as it is known to be expressed in the RPE, and here, too, we detected a significant reduction (−3.9 FC, P<9×10^{-4}).

High-throughput transcriptome analysis of Dicer1-deficient RPE and identification of miRNA-dependent gene networks in the developing RPE

Since miRNAs generally function as regulators of mRNA stability (Guo et al., 2010), we utilized Affymetrix GeneChip Mouse Gene 1.0 ST arrays to identify transcript changes following *Dicer1* deletion. From an input of 28,853 genes, we identified 318 that increased in expression upon *Dicer1* deletion and 212 that decreased in expression (FC>1.5, P<0.05 corrected for FDR<0.1; supplementary material Table S2; GEO accession GSE69883). Among the downregulated genes were factors involved in the visual perception pathway, namely *Rpe65* and *Lrat*, which is consistent with their decreased expression by immunostaining (Fig. 4). In addition, reduced levels of expression of mRNAs encoding proteins associated with melanosome biogenesis (*MlanA*, *Gpr143*) and cell adhesion (*Cdh4*, *Itga11*) were observed, in agreement with reduced pigmentation and altered morphology of the *Dicer1* mutant RPE (Fig. 3). Moreover, increased levels of *Pax6* and *Meis2* were detected, possibly reflecting abrogated maturation of the Dicer1-deficient RPE.

We next used an *in silico* approach to identify the putative mRNA targets for miRNAs, focusing on mRNAs that were significantly upregulated in Dicer1-deficient RPE and that are putative targets of miRNAs strongly downregulated in Dicer1-deficient RPE. The analysis revealed 26 putative target mRNAs for miR-20b/106a, seven for miR-221/222 and 14 for miR-204/211 (Fig. 8; supplementary material Table S3). As miR-204 is highly expressed in the embryonic and adult RPE and is considered to play an important role in RPE differentiation based on functional studies in primary culture (Zhao et al., 1997), we focused on examining the expression *in vivo*, in

Development (2015) 142, 2487-2498 doi:10.1242/dev.121533

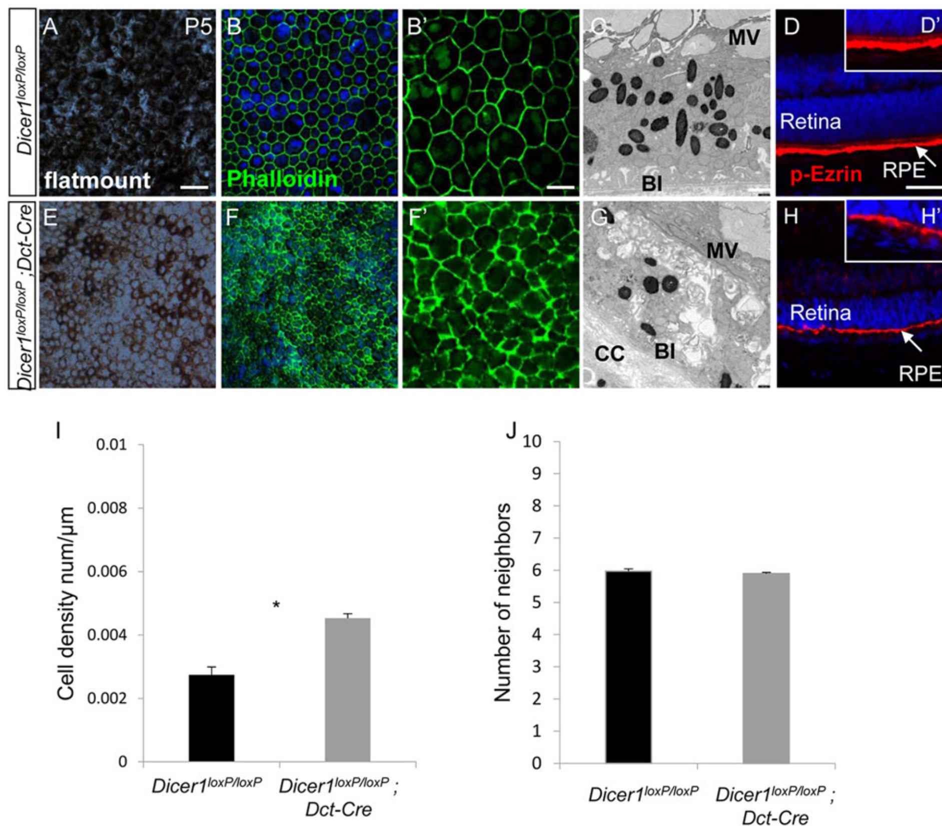

Fig. 3. Dicer1 is required for normal pigmentation and adhesive properties in the RPE. (A-H′) *Dicer1$^{loxP/loxP}$* (top row) and *Dicer1$^{loxP/loxP}$;Dct-Cre* (bottom row) RPE at P5 viewed in flat-mount by bright-field (A,E) and following phalloidin staining (B,B′,F,F′), TEM (C,G) and immunostaining against phospho-ezrin (D,D′,H,H′). Apical microvilli are shorter in the mutant RPE (G,H,H′) than in control RPE (C,D,D′). Insets show higher magnifications. (I) Quantification of cell density reveals a significant increase in the *Dicer1$^{loxP/loxP}$;Dct-Cre* RPE (*P<0.02, n=6). (J) Quantification of average number of neighboring cells shows no significant difference (P>0.3, n=6). Error bars indicate s.e. BI, basal infoldings; CC, choriocapillaris; MV, microvilli. Scale bars: 100 µm in A,B,D,E, F,H; 20 µm in B′,F′; 0.5 µm in C,G.

control and *Dicer1* mutant RPE, of predicted miR-204 targets that showed altered expression in the microarray analysis. *Meis2*, which is a key developmental regulator and miR-204 target in the eye (Conte et al., 2010), and Pax6 were elevated based on the microarray analysis (2.13 and 4.03 FC, respectively) and this was confirmed on a protein level by antibody labeling (supplementary material Table S2 and Fig. S4). The decrease in *Slc16a8* (−2.6 FC), which was recently found to be altered in AMD (Adijanto et al., 2012), and the increase in *Ap1s3* (1.77 FC), which is involved in clathrin-mediated vesicular transport from the Golgi or endosome (Boehm and Bonifacino, 2002), were validated by QPCR conducted on *Dicer1* mutant and control RPE cells (supplementary material Fig. S4).

To gain a comprehensive and unbiased view of the miRNA-dependent gene network in the developing RPE, we combined a String protein-protein interaction analysis (http://string-db.org; Franceschini et al., 2013), which was used to assemble the predicted networks of the significantly altered genes (FC>1.5; P<0.05 corrected for FDR<0.1), with the functional categories highlighted by gene ontology (GO) analysis (DAVID, http://david.abcc.ncifcrf.gov; see Tables 2 and 3) and with miRNA target predictions (using Partek Genomics Suite) for the four miRNA families that were downregulated in the Dicer1-deficient RPE (miR-20b/106a, miR-221/222, miR-204/211, miR-155; Figs 1 and 7). This integrated analyses revealed a complex network of genes and miRNAs involved in RPE development and predicted to participate in regulating RPE differentiation and function (Fig. 8). Importantly, it was evident that changes in the expression of miRNA families could generate an exceedingly complex RPE transcriptome output. Some of the predicted targets appeared to be regulated by several miRNAs (e.g. *Csrnp3* is a predicted target of miR-204 and miR-20b) and, in turn, participated in multiple biological functions including cell adhesion, cell motion and EMT. This analysis emphasizes the regulatory power and efficacy of miRNA and points to key players that mediate the normal differentiation and function of the RPE.

DISCUSSION

Dicer1 is dispensable for RPE fate and survival during development

During the early stages of vertebrate eye development, the optic neuroepithelium is patterned to neuroretina, pigmented epithelium or optic stalk lineages that eventually populate the inner, outer and ventral OC, respectively (Chow and Lang, 2001; Fuhrmann, 2010). The specified pigmented progenitors initially maintain the potential to transdifferentiate into neuronal fate, as observed following surgical damage to the OC or as a result of a single morphogene or transcription factor disruption. This transdifferentiation potential has been extensively studied in the context of regenerating the retina from the pigmented OC derivatives: RPE, ciliary body and iris (Luz-Madrigal et al., 2014; Tropepe et al., 2000; Wohl et al., 2012). miRNAs are central regulators of developmental processes including tissue regeneration (Song et al., 2010a; Thatcher et al., 2008) and several miRNAs have been documented to play key roles in maintaining cell fate in multiple lineages, as shown for miR-142 in the generation of hematopoietic stem cells and for miR-375 in the endocrine beta-cell lineage (Kaspi et al., 2014; Nimmo et al., 2013; Poy et al., 2004). Considering these central roles of miRNAs and the developmental potential of the embryonic RPE, it is intriguing that the deletion of *Dicer1* in the specified pigmented cells did not result in any overt change in RPE cell identity based on the expression pattern of key RPE and retinal genes (*Sox9*, *Otx2*, *Mitf*, *Chx10*), the gross morphology of the single cell layer and the morphology of the RPE cells (Figs 1-3). The maintenance of cell fate was further corroborated by the transcription profile of the Dicer1-deficient RPE, which did not expose any significant elevation in the

Development (2015) 142, 2487-2498 doi:10.1242/dev.121533

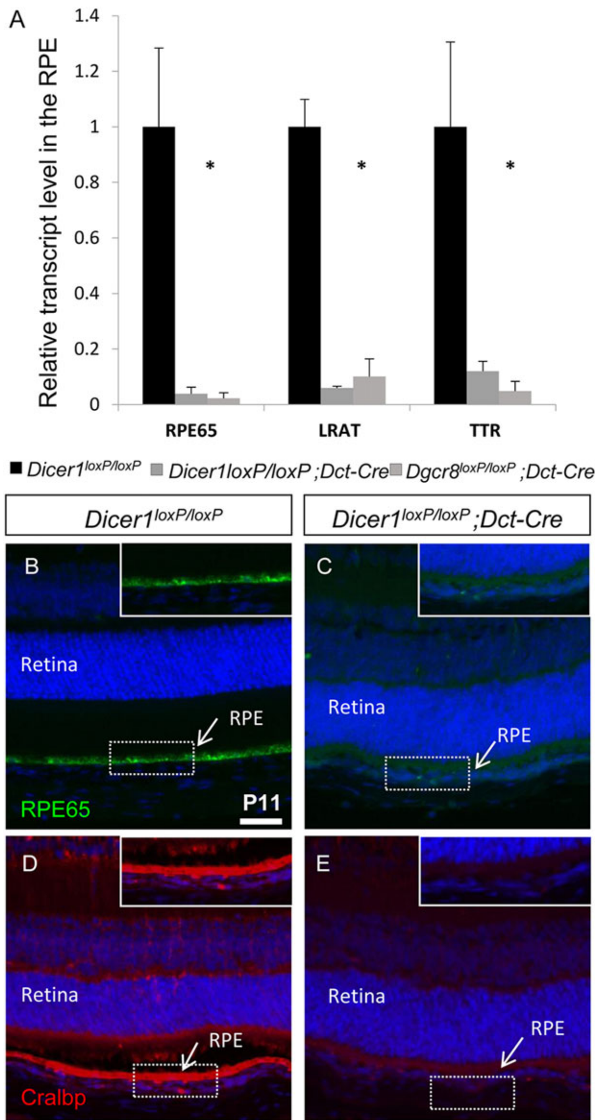

Fig. 4. Ablation of *Dicer1* in the RPE results in downregulation of visual cycle components. (A) QPCR for monitoring the expression of visual cycle genes at P5, showing significant reduction of *Rpe65*, *Lrat* and *Ttr* transcripts in the *Dicer1*- and *Dgcr8*-deficient RPE. *P<0.05, n=6, error bars indicate s.d. (B-E) Immunofluorescence against Rpe65 and Cralbp proteins in control (B,D) and *Dicer1$^{loxP/loxP}$;Dct-Cre* mutant (C,E) RPE at P11. Boxed regions are magnified in insets. Scale bar: 50 µm.

expression of neuronal genes. Thus, at the stage at which miRNAs are depleted from the *Dicer1$^{loxP/loxP}$;Dct-Cre* OC, the RPE fate is acquired and maintained through miRNA-independent mechanisms.

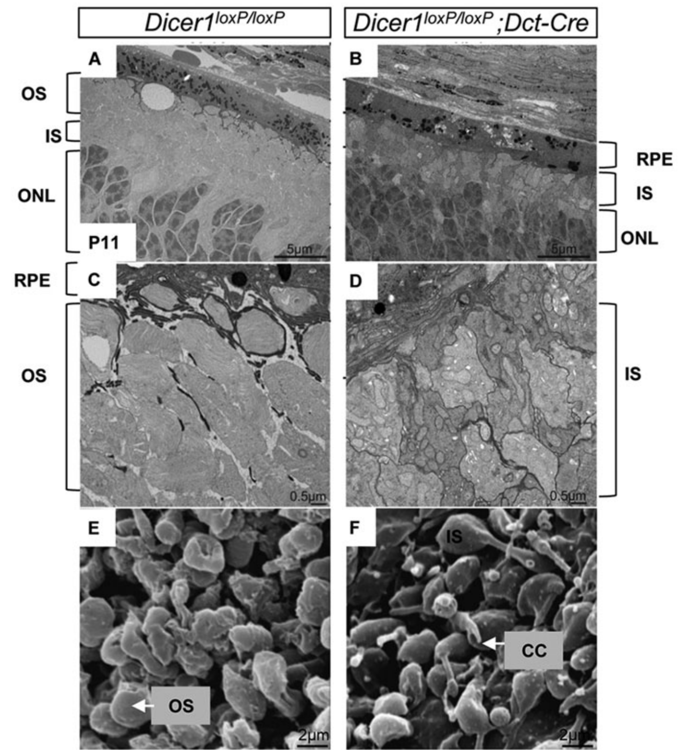

Fig. 5. Dicer1-deficient RPE fails to develop photoreceptor OSs. TEM (A-D) and SEM (E,F) images showing the ultrastructure of the RPE and photoreceptors from P11 *Dicer1$^{loxP/loxP}$* control (A,C,E) and *Dicer1$^{loxP/loxP}$; Dct-Cre* (B,D,F) mice. cc, connecting cilia; IS, inner segment; ONL, outer nuclear layer; OS, outer segment; RPE, retinal pigmented epithelium. Scale bars: 5 µm in A,B; 0.5 µm in C,D; 2 µm in E,F.

It is interesting to note that, although basic cell identity is preserved despite miRNA loss, there was an increase in cell density that corresponded with the reduction in cell size (Fig. 3). The increase in cell density might also reflect elevated proliferation, although this is less likely as we did not detect elevation in Ki67 and the *Dicer1$^{loxP/loxP}$;Dct-Cre* eyes were smaller than normal (data not shown). We therefore conclude that miRNAs are required for normal growth of the RPE cells, which is reminiscent of recent findings concerning roles of miRNAs in regulating the growth of cardiomyocytes (Song et al., 2010b).

In the adult RPE, Dicer1 is considered to be a survival factor as it is primarily required for the removal of toxic double-stranded RNA emanating from transposable elements, while the contribution of miRNAs to RPE physiology and survival remains questionable (Kaneko et al., 2011). In contrast to the findings on Dicer1 in the adult RPE, the results presented in this study reveal that, during RPE development, the role of Dicer1 in miRNA biogenesis is cardinal for

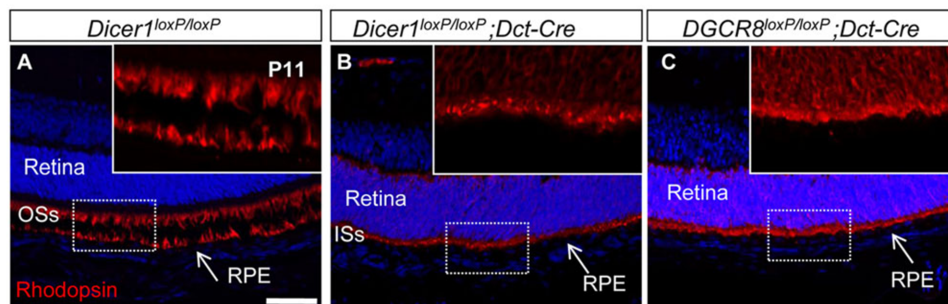

Fig. 6. Rhodopsin accumulation in *Dicer1$^{loxP/loxP}$;Dct-Cre* and *Dgcr8$^{loxP/loxP}$; Dct-Cre* photoreceptors. (A) Rhodopsin (red) is abundant in photoreceptor OSs in control RPE. (B,C) In the *Dicer1$^{loxP/loxP}$;Dct-Cre* RPE and *Dgcr8$^{loxP/loxP}$;Dct-Cre* RPE, most of the rhodopsin has been mistargeted to the outer nuclear layer. ISs, inner segments; OSs, outer segments; RPE, retinal pigmented epithelium. Boxed regions are magnified in insets. Scale bars: 100 µm.

Development (2015) 142, 2487-2498 doi:10.1242/dev.121533

Table 1. miRNAs significantly decreased upon RPE-specific Dicer1 depletion

miRNA	FC	*P*-value
miR-344*	−19.39	1.94×10^{-6}
miR-204*	−11.16	1.45×10^{-5}
miR-9-star	−3.637	1.69×10^{-3}
miR-181a-2-star	−2.971	2.98×10^{-3}
miR-20b*	−2.928	1.17×10^{-4}
miR-501-5p	−2.544	3.40×10^{-3}
miR-211	−2.539	8.76×10^{-3}
miR-1839-3p	−2.435	0.0117
miR-155	−2.191	0.0202
miR-34a	−2.086	0.0181
miR-138	−2.031	2.12×10^{-3}
miR-708*	−2.025	8.52×10^{-5}
miR-222*	−1.865	3.69×10^{-4}
miR-363	−1.829	3.53×10^{-3}
miR-106a*	−1.816	5.91×10^{-4}
miR-339-5p	−1.674	0.0230

FC, fold change for *Dicer1* mutant versus control. *FDR<0.1.

tissue maturation and function. This conclusion is drawn from the following observations. First, the phenotype of *Dgcr8*$^{loxP/loxP}$;*Dct-Cre* mimicked that of *Dicer1*$^{loxP/loxP}$;*Dct-Cre* in terms of RPE and photoreceptor morphology, expression of visual cycle genes and cell survival (Figs 4 and 6; supplementary material Fig. S3), indicating that the phenotype is due to loss of miRNAs rather than any alternative activities of Dicer1. Second, we did not detect any increase of apoptosis in the *Dicer1* mutant RPE during embryonic and early postnatal development (P11, supplementary material Fig. S3) nor any accumulation of Alu-like B1 sequences in the P5 *Dicer1* mutant RPE (not shown). Together, these findings support the notion that, during RPE development, Dicer1 activities are primarily related to the biogenesis of miRNAs rather than to the degradation of toxic RNA.

An intriguing finding was that survival of the RPE was not drastically compromised despite loss of miRNAs. This was surprising, especially considering the key role attributed to miRNAs in the survival of neural crest-derived melanocytes (Levy et al., 2010). In the melanocyte lineage, Mitf controls the expression of Dicer1, which in turn enables the biogenesis of the miR-17-92 cluster, which downregulates the pro-apoptotic factor Bcl2l11 (Bim) (Fontana et al., 2008; Levy et al., 2010; Ventura et al., 2008). Reminiscent of these findings in melanocytes, in the Dicer1-deficient RPE we also detected upregulation of *Bcl2l11* (1.58 FC, $P<0.001$) as well as the downregulation of miR-20b and miR-106a (Table 1), which have the same seed sequence as miR-17 and thus could regulate Bcl2l11 levels (Ventura et al., 2008). The alteration in expression of Bcl2l11 was, however, insufficient to trigger RPE cell death during developmental stages. This suggests that RPE is more resilient to alterations in the expression of proapoptotic factors than the melanocyte lineage. It would be interesting in future studies to examine further the involvement of miR-20b and miR-106a in the regulation of Bcl2l11 in the RPE and also the potential differences between the RPE and melanocyte lineages with respect to the mechanisms that have evolved to assure tissue survival.

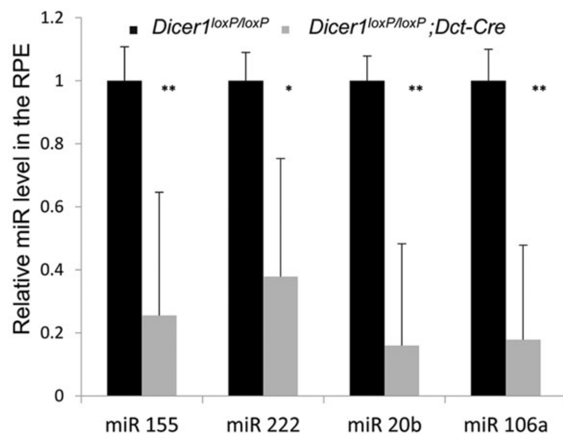

Fig. 7. miRNAs significantly downregulated as a consequence of RPE-specific Dicer1 depletion. Expression of mature miRNAs in RPE of P5 *Dicer1*$^{loxP/loxP}$ and *Dicer1*$^{loxP/loxP}$;*Dct-Cre* mice was analyzed by QPCR with TaqMan probes. miRNA expression levels were quantified in comparison with U6 RNA (*Rnu6*) as the endogenous control. All miRNAs analyzed were significantly downregulated in the *Dicer1*$^{loxP/loxP}$;*Dct-Cre* RPE. *$P<0.05$, **$P<0.005$, $n=6$, error bars indicate s.d.

Multiple miRNA families are required for the execution of RPE differentiation programs

Roles for miRNAs in lineage maturation have been documented in many tissues, including retinal, renal, endocrine and Schwann cell development (Kaspi et al., 2014; Patel et al., 2012; Verrier et al., 2010). Consistent with these findings, our study reveals that miRNAs are essential for execution of the RPE differentiation programs, based on the reduced expression of genes involved in the visual cycle and cell adhesion (Fig. 8). The loss of visual cycle genes was most striking, as most of the pathway genes were downregulated in the Dicer1-deficient RPE (Fig. 4; DAVID GO analysis, $P<3.5\times10^{-5}$, Table 2, Fig. 8). Moreover, the expression of the three transcription factors (Sox9, Lhx2 and Otx2) that were recently documented to regulate the visual cycle genes (Masuda et al., 2014) was maintained in the Dicer1-deficient RPE (Fig. 2). A possible explanation for the differentiation arrest is the observed abnormal upregulation of *Otx1*, *Pax6* and *Meis2* (FC 2.2, 4 and 2.1, $P<0.01$; supplementary material Fig. S4), which are normally detected during embryonic stages but not in postnatal RPE (Hever et al., 2006; Martinez-Morales et al., 2001). The misexpression of Pax6 and Meis2, however, was restricted to the OC periphery, which normally differentiates later than the central OC and therefore the increase in expression of these factors might reflect a delay in tissue maturation rather than accounting for the loss of visual cycle gene expression throughout the RPE.

It is currently unclear which miRNAs underlie the arrest in RPE maturation, although several of the miRNAs identified in the embryonic RPE in the course of this study have been implicated in tissue maturation. miR-20b/106a and miR-222/221 families are also likely to have a key role in RPE differentiation and physiology. These miRNAs have been shown to play important roles in physiological functions such as EMT and in pathologies such as cancer (Saleiban et al., 2014; Stinson et al., 2011).

Probably the most extensively studied miRNA family in the context of RPE differentiation is miR-204/miR-211, which are highly enriched in the RPE and miR-204 was previously documented to play role in the RPE lineage using cultured cells (Wang et al., 2010). To identify its contribution to the phenotype of Dicer1-deficient RPE, we performed *in silico* analysis that uncovered 14 putative targets of miR-204/211 that were upregulated in the Dicer1-deficient RPE (Fig. 8; supplementary material Table S3). Among these targets, several were noted as important developmental regulators (*Meis2*) (Conte et al., 2010; Xu et al., 2002) as well as encoding proteins involved in RPE physiology, such as *Slc16a8* (Adijanto et al., 2012). Slc16a8 is a member of a family of proton-

Development (2015) 142, 2487-2498 doi:10.1242/dev.121533

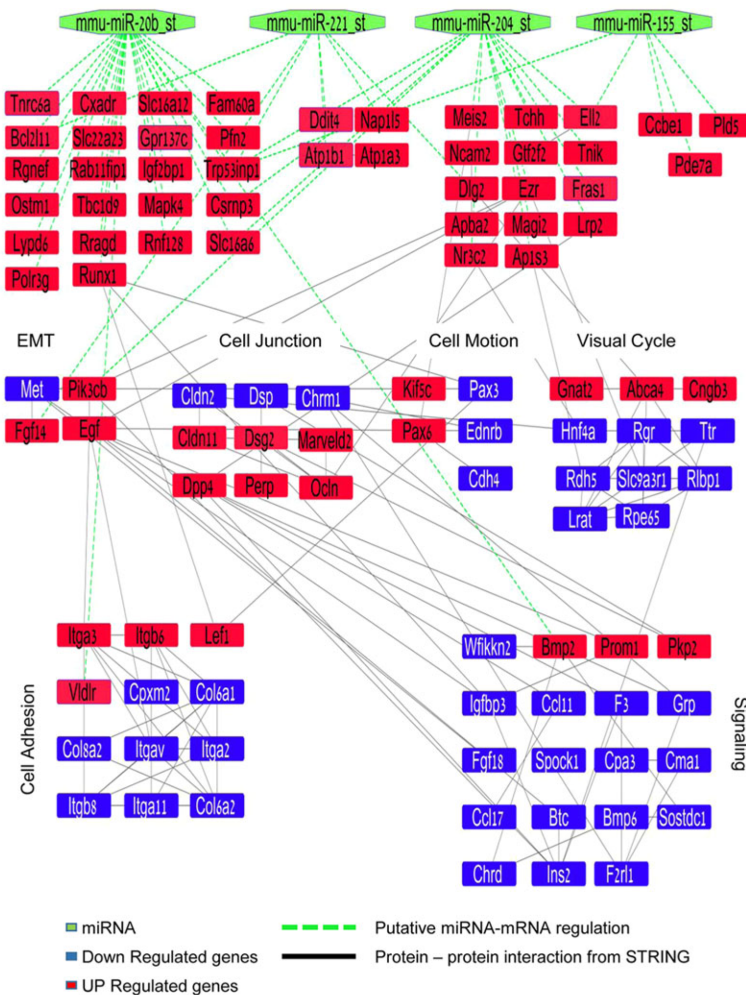

Fig. 8. miRNA-dependent gene networks in the developing RPE. Summary network showing the significantly downregulated miRNAs (green), upregulated (red) and downregulated (blue) genes [FC>1.5 (upregulated) or FC<−1.5 (downregulated), P<0.05 corrected for FDR<0.1 or validated by QPCR] in the mutant RPE. The green dashed lines connect miRNAs to their targets. The black lines indicate protein-protein interactions reported by the String analysis. The DAVID GO annotation was used to cluster the genes by biological function.

coupled monocarboxylate transporters that mediate lactate transport across the cell membrane, and Slc16a8 is highly expressed in the RPE, required for visual function and associated with AMD (Daniele et al., 2008; Philp et al., 1998; Priya et al., 2012), and has been experimentally shown to be upregulated by miR-204 (Adijanto et al., 2012). Genes with as yet unknown functions in the RPE, such as *Ap1s3*, which encodes a subunit of adaptor protein complex, should also be considered when attempting to identify the underlying cause of the phenotype following miRNAs loss from the RPE, as this subunit was recently shown to play a role in the endosomal translocation of signaling components involved in inflammation in skin keratinocytes (Boehm and Bonifacino, 2002; Setta-Kaffetzi et al., 2014).

Previous studies conducted in cultured human RPE cells suggested that miR-204 is essential for the maintenance of RPE specification, as without miR-204 the cells lose their epithelial identity and undergo EMT (Adijanto et al., 2012). By contrast, it seems that *in vivo* EMT does not take place in the absence of miRNAs including miR-204. This discrepancy might be caused by differences in the experimental design. The ablation of miR-204 *in vivo* was induced after RPE specification, whereas in the culture the inhibition of miR-204 was triggered in the dedifferentiation state (Adijanto et al., 2012). Also, *in vivo*, the adjacent structures of the eye, which are genotypically normal, might mechanically contribute to preventing the alteration in cell morphology. EMT might also be inhibited in the Dicer1-deficient RPE due to the abrogated expression of miRNAs and mRNA required for this process. It is known that certain miRNAs are upregulated during EMT, such as miR-27 (Bullock et al., 2012); however, as the expression of pro-EMT miRNAs is prevented in the Dicer1-deficient RPE, thus factors required for full EMT might be absent from the Dicer1-deficient cells.

Table 2. DAVID GO analysis of significantly downregulated genes

Function	Count	P-value	Enrichment score
Signal	64	6.7×10^{-9}	8.62
Cell adhesion	20	1.8×10^{-8}	5.75
Cell junction	20	2.9×10^{-8}	2.68
Cell motion	16	4.1×10^{-5}	2.2
Visual perception	9	3.1×10^{-5}	2

DAVID GO analysis of downregulated genes: FC<−1.5, P<0.05 corrected for FDR<0.1. The enrichment score is according to Huang et al. (2007).

Table 3. DAVID GO analysis of significantly upregulated genes

Function	Count	P-value	Enrichment score
Signal	124	8.1×10^{-12}	9.02
Cell adhesion	30	3.5×10^{-5}	4.06
Cell junction	26	4.5×10^{-6}	3.48
Ion transport	39	9.0×10^{-7}	2.44

DAVID GO analysis of upregulated genes: FC>1.5, P<0.05 corrected for FDR<0.1. The enrichment score is according to Huang et al. (2007).

Development (2015) 142, 2487-2498 doi:10.1242/dev.121533

OS morphogenesis and photoreceptor survival require miRNA function in the RPE

The current study demonstrates a specific requirement for miRNAs in the RPE for proper OS morphogenesis and photoreceptor survival. During normal photoreceptor differentiation the OS extends from the basal end of the connecting cilia through the evagination of membranes at their apical end. The formation of new disk membranes continues throughout life as a component of OS renewal and is dependent on multiple ciliary factors that are required for protein and vesicular trafficking and fusion (Ramamurthy and Cayouette, 2009; Wheway et al., 2014). As the connecting cilia formed properly in the Dicer1-deficient RPE (supplementary material Fig. S2) it seems that the observed photoreceptor developmental arrest is due to a failure to initiate the assembly of the OS disk membranes.

The absence of the OS was detected throughout the entire *Dicer1* and *Dgcr8* mutant retina, although most photoreceptors were of normal genotype (supplementary material Fig. S1C). These results support a non-cell-autonomous requirement of miRNAs in the RPE for proper OS morphogenesis. The requirement of RPE for photoreceptor maturation was previously noted in studies employing methods to ablate the RPE *in vivo* or in explants (Hollyfield and Witkovsky, 1974; Raymond and Jackson, 1995). This is further supported by more recent reports that the majority of photoreceptors produced from embryonic or induced pluripotent stem cells in culture fail to develop OSs (Meyer et al., 2009; Osakada et al., 2008; Zhong et al., 2014). This differentiation failure might be because RPE is not maintained in these cultures. Interestingly, when mouse photoreceptor precursors are transplanted into the subretinal space immediately next to the RPE layer, the grafts undergo lamination and develop OSs (Tucker et al., 2011). Furthermore, it has been shown in culture that RPE-conditioned medium is sufficient to promote OS growth (Gaur et al., 1992). In addition, a recent study revealed for the first time that DNA methyltransferase (*Dnmt1*) knockdown in RPE results in aberrant photoreceptor development and lack of OS morphogenesis (Nasonkin et al., 2013). Based on our microarray analysis, we did not observe downregulation of *Dnmt1* in the Dicer1-deficient RPE. Therefore, it would be interesting to investigate whether well-known miRNAs in the RPE, such as miR-204, or other miRNAs identified in the course of this study are downregulated in the Dnmt1-deficient RPE and mediate the lack of OS development. These findings point to a key role of the RPE in photoreceptor maturation and generation of the OS, the crucial structure for phototransduction. Resolving the contribution of miRNAs of the RPE to this process will have important clinical implications for designing cellular replacement therapies for photoreceptors and RPE.

In conclusion, the network analysis reveals a complex gene regulatory network that encompasses a few miRNA families (miR-204/211, miR-222/221, miR-20b/106a; Fig. 8). The RPE miRNAs seem to be dispensable for the specification and survival of the embryonic RPE at the OC stage, yet their combined activity is essential for the correct expression of multiple genes required for normal differentiation and physiology of the RPE and adjacent photoreceptors.

MATERIALS AND METHODS

Mouse lines

The mouse lines employed were: *Dct-Cre* (Davis et al., 2009) *Dicer1^{loxP}* (Harfe et al., 2005), *Dgcr8^{loxP}* (Yi et al., 2009) and *Z/EG* (Novak et al., 2000). The *Dct-Cre* mice were crossed with the other lines to establish *Z/EG;Dct-Cre*, *Dicer1$^{loxP/loxP}$;Dct-Cre* and *Dgcr8$^{loxP/loxP}$;Dct-Cre* somatic mutants. Littermates lacking *Dct-Cre* were used as controls. All animal work was conducted according to national and international guidelines and approved by the Tel Aviv University review board.

Immunofluorescence, *in situ* hybridization (ISH) and preparation of flat-mounts

Immunofluorescence analysis was performed as described previously (Ashery-Padan et al., 2000) with the antibodies listed in supplementary material Table S5.

Lineage tracing and quantification of Cre activity in *Z/EG;Dct-Cre* was by immunodetection of GFP on paraffin sections. The percentage of GFP-expressing cells in the peripheral or central photoreceptor layer was calculated from four eyes.

ISH was performed on 14 µm cryosections using DIG-labeled RNA probe for *Trpm3* as previously described (Yaron et al., 2006; Shaham et al., 2013). For miRNA ISH, hsa-miR-204 and mmu-miR-211 miRCURY LNA detection probes (working concentration 20 nM; Exiqon, 88076-15) were hybridized to frozen sections as described previously (Xu et al., 2007).

Flat-mount samples were prepared from P5 eyes, which were fixed in 4% paraformaldehyde for 30 min followed by separation of the RPE, which was then flattened on membrane filters (Schleicher & Schuell, 0.45 µm, D-37582) and processed for phalloidin staining (1:100; Invitrogen, A12379).

Cells were counted and normalized to area to determine cell density. Quantification of the number of neighboring cells was performed for ten randomly chosen cells in each defined area.

Scanning electron microscopy (SEM)

Eyes were fixed overnight in 0.1% cacodylate-buffered fixative containing 2.5% paraformaldehyde and 2.5% glutaraldehyde. After 30 min incubation in 1% osmium tetroxide and dehydration in ascending ethanol and acetone series, specimens were critical-point dried, sputter-coated with gold and examined under a JSM 840A scanning electron microscope.

Transmission electron microscopy (TEM)

Mouse eyes were fixed in 0.1 M cacodylate-buffered fixative containing 2.5% paraformaldehyde and 2% glutaraldehyde. After dissection, samples were rinsed in 0.1 M cacodylate buffer, postfixed in a mixture of 1% osmium tetroxide and 0.8% potassium ferrocyanide in 0.1 M cacodylate buffer for 2 h at 4°C. Specimens were then dehydrated in a graded series of ethanol and embedded in Epon (Serva). Ultrathin sections were mounted on uncoated copper grids, stained with uranyl acetate and lead citrate and examined on a Zeiss EM 10A electron microscope.

Microarray analysis

Global miRNA expression and gene expression data were determined using Affymetrix miRNA 2.0 arrays and Affymetrix GeneChip Mouse Gene 1.0 ST arrays, respectively. Three independent biological repeats were performed for P5 controls versus *Dicer1$^{loxP/loxP}$;Dct-Cre*. The microarray analysis was performed in the Microarray Unit of the Cancer Research Center, Sheba Medical Center according to the manufacturer's procedure (Affymetrix). Microarray data analysis and miRNA putative target analysis were performed using Partek Genomics Suite (Partek). The values presented in supplementary material Table S1 and Table S2 for the differentially expressed miRNA and mRNA are corrected for multiple testing, with *P*-values lower than 0.05 corrected for FDR<0.1 and with an FC cut-off of 1.5. The String algorithm was employed using the default settings (http://string-db.org/).

Quantitative real-time PCR (QPCR)

For QPCR analysis of mature miR-204, miR-155, miR-106a, miR-222 and miR-20b, 2.5 µl 4 ng/µl total RNA was used for synthesis of first-strand cDNA in a MultiScribe reverse transcriptase reaction with the High Capacity cDNA Kit (Applied Biosystems) and TaqMan MicroRNA Assay RT primers (Applied Biosystems). The comparative Ct method ($2^{-\Delta\Delta Ct}$) was used to calculate relative expression as described previously (Chen et al., 2005).

For QPCR analysis of mRNA, reverse transcription of 1 µg RNA from each sample was performed using the SuperScript III First Strand Kit (Invitrogen). cDNA was amplified using the Power SYBR Green Mix (Applied Biosystems) in a 384-well optical reaction plate using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). Results were

calibrated in relation to an average of two house-keeping genes, *Ppia* and *Tbp*, after verifying that their levels were consistent in normal and mutant RPE. Raw data were processed using the comparative Ct method. Each amplification reaction was performed in triplicate using 20 ng cDNA for each sample. Primers are listed in supplementary material Table S4.

Statistical analysis

The statistical significance of differences between mean values of two groups was determined using Student's *t*-test. $P<0.05$ was considered significant.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceived and designed the experiments: R.O., B.W.-K. and R.A.-P. Performed the experiments: R.O., B.W.-K., S.R., E.R.T., A.S.S. and A.R. Analyzed the data: R.O., B.W.-K., E.R.T., A.S.S., D.N., R.S., M.P.-C. and R.A.-P. Wrote the paper: R.O., B.W.-K. and R.A.-P.

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Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.121533/-/DC1

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