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microRNA expression in the eyes and their significance in relation to functions

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A B S T R A C T

microRNAs (miRNAs) are endogenous, small, non-coding, regulatory RNAs, ~22 nucleotides (nts) in size. Since the first discovery of miRNAs in 1993 in *Caenorhabditis elegans*, miRNAs have been shown to be widely expressed in metazoans and plants in tissue-specific and developmental stage-specific manners. miRNAs target their downstream messenger RNAs (mRNAs) by base pairing to their target sites with sequence complementarity, mainly in the 3′ untranslated region (UTR), and induce the breakdown of the targeted mRNAs and/or inhibition of translation from the mRNAs. Approximately 30% of the protein-coding genes are estimated to be regulated by miRNAs. One miRNA can target hundreds of downstream target mRNAs, while one mRNA can be targeted by multiple miRNAs. miRNAs have been recognized as a major level of post-transcriptional regulation of the fine-tuning of gene expression, playing important roles in cellular proliferation, differentiation, and cell death and are involved in all aspects of the biological processes investigated thus far. Mutations in miRNAs and/or the target sites in the transcripts of their downstream target genes and dysregulation of miRNA biogenesis can result in a wide variety of diseases, including cancers.

In the past few years, we and other groups have investigated miRNA expression in the retina and other ocular tissues. The miRNA transcriptomes of the retina, lens, and cornea have been established. Many miRNAs showed unique tissue-specific and developmental stage-specific expression patterns, suggesting potential unique functions in the retina and other ocular tissues. In spite of this progress, the roles of retinal and other ocular miRNAs in the development, normal functions, and diseases of the retina and other ocular tissues are still largely unknown. This review aims to provide an extensive summary of the current status and recent progress of our understanding of the miRNAs in retinal and other ocular tissues and speculate on future directions to define the roles of miRNAs in the retina and other ocular tissues and diseases.

Contents

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1. Introduction – discovery, biogenesis, and functions of miRNAs

microRNAs (miRNAs) are newly recognized, non-coding, regulatory RNA molecules, about 22 nucleotides (nts) in length, and found in all metazoans studied thus far (Bartel, 2004). miRNAs were first discovered in 1993 in *Caenorhabditis elegans* through classical forward genetics analyzing the two key *C. elegans* mutants, *lin-4* (Horvitz and Sulston, 1980) and *lin-14* (Ferguson et al., 1987). Ambros group (Lee et al., 1993) and Ruvkun group (Wightman et al., 1993) discovered that *lin-4* is a non-protein-coding gene and that two small *lin-4* transcripts of ~22 and 61 nts of *lin-4* contain sequences complementary to the 3′UTR of *lin-14* mRNA and negatively regulate the level of the *lin-14* protein. This negative regulation of *lin-14* by *lin-4* created a temporal decrease of the *lin-14* protein starting from the first larval stage and plays an important role in the temporal pattern formation through sequential differentiation in *C. elegans*. The shorter *lin-4* RNA is now recognized as the founding member of miRNAs (Bartel, 2004). Since then, miRNAs have been identified in almost all metazoans and plants (Aravin et al., 2003; Hamilton and Baulcombe, 1999; Lagos-Quintana et al., 2001, 2002, 2003; Lau et al., 2001). According to the miRBase database (Aug 2007, release10.0. http://microrna.sanger.ac.uk/sequences), at least 85, 135, 455, and 546 miRNAs have been experimentally verified in *Drosophila*, *C. elegans*, mouse, and human genomes, respectively (Griffiths-Jones et al., 2008). It is estimated that the human genome may have 800–1000 miRNAs (Bentwich et al., 2005). Recently, miRNAs also have been identified in unicellular green alga, *Chlamydomonas reinhardtii*, (Molnar et al., 2007; Zhao et al., 2007a), suggesting that the miRNA pathway is an ancient mechanism of gene regulation that evolved prior to the emergence of multicellular organisms.

Most of the miRNA genes are transcribed by RNA polymerase II (Pol II) (Borchert et al., 2006; Cai et al., 2004; Y. Lee et al., 2004). Many miRNA genes have their own promoters and transcriptional regulatory elements to ensure their tissue-specific and developmental stage-specific expression. The primary transcripts of the miRNA genes, referred to as pri-miRNAs, are spliced and have a 5′ 7-methylguanosine cap (^{m7}G) and a poly(A) tail (Cai et al., 2004; Y. Lee et al., 2004). In the nucleus, the pri-miRNAs fold into hairpin structures that are cleaved by an RNase III endonuclease, Drosha in the Drosha–DGCR8 complex (Han et al., 2004, 2006), to form 60–70-nt stem loop intermediates, known as pre-miRNAs, with a 2-nt 3′ overhang (Y. Lee et al., 2003, 2004; Zeng et al., 2003) (Fig. 1).

More than 100 known miRNAs are located in the introns of protein-coding genes (Rodriguez et al., 2004). They are spliced out from the primary transcript and are processed by Drosha to pre-miRNAs. Some spliced-out introns in *C. elegans* and mammals correspond precisely to pre-miRNA, mimic the structural features of the pre-miRNAs, and enter the downstream miRNA-processing pathway without Drosha-mediated cleavage (Ruby et al., 2007).

Approximately 20% of human miRNAs may be derived from repetitive sequences and transcribed by RNA polymerase III (Borchert et al., 2006; Smalheiser and Torvik, 2005).

Pre-miRNAs are transported to cytoplasm by Ran-GTP and export receptor, Exportin 5 (Lund et al., 2004; Yi et al., 2003). In the cytoplasm, pre-miRNAs are cleaved by another RNase III endonuclease, Dicer (E.J. Lee et al., 2003; Y.S. Lee et al., 2004), in the Dicer/TRBP (TAR RNA-binding protein) complex, to form ~22-bp miRNA duplexes with 2-nt 3′ overhang, characteristic of RNase III-mediated cleavage (Hutvagner et al., 2001; Ketting et al., 2001). TRBP recruits Argonaute protein Ago2 and other Ago proteins to initiate the assembly of the RNA-induced silencing complex (RISC) (Chendrimada et al., 2005; Gregory et al., 2005). Only one strand of the duplex becomes mature miRNA and incorporated in the RISC; the other strand, designated as miRNA*, is degraded. The miRNA strand with a relatively lower thermodynamic stability of base pairing at its 5′ end is usually selected to become mature miRNA (Du and Zamore, 2005; Schwarz et al., 2003). Argonaute family proteins are the key components of the RISC complex. In mammals, there are four Ago proteins, Ago1–Ago4 (Peters and Meister, 2007). Ago2 is the one that has RNase H-like endonucleolytic activity (Liu et al., 2004; Meister et al., 2004).

Mature miRNAs base pair with target mRNAs in the 3′UTR. When the miRNA and the target sites have precise or nearly precise complementarity, miRNAs (with the RISC) specify cleavage of the target mRNAs by Ago2 (Rhoades et al., 2002); when the base pairing is imperfect, miRNA (with the RISC) can destabilize the mRNA by deadenylation and subsequent decapping (Giraldez

Nomenclature

AANAT	arylalkylamine *N*-acetyltransferase
Adcy6	adenylyl cyclase VI
adRP	autosomal dominant retinitis pigmentosa
AMD	age-related macular degeneration
BAG4	*BCL2*-associated athanogene 4
BCL2	B-cell CLL/lymphoma 2
BHLHB3	basic helix–loop–helix domain containing, class B, 3
CEBPA	CCAAT/enhancer-binding protein, alpha
CGZ	circumferential germinal zone
CKIϵ	Casein kinase I epsilon
CMZ	ciliary margin zone
CNS	central nervous system
CT	circadian time
DAVID	database for annotation, visualization and integrated discovery
DCX	doublecortin
Dec2	differentially expressed in chondrocytes protein 2
DHCR24	24-dehydrocholesterol reductase
DR	diabetic retinopathy
DRG	dorsal root ganglia
DTR	diphtheria toxin receptor
EBF	early B-cell factor
EGFP	enhanced green fluorescent protein
EGFR	EGF receptor
ERG	electroretinogram
ERK	extracellular signal-related kinase
FAIM	Fas apoptotic inhibitor molecule
FMR1	fragile X mental retardation 1
FMRP	Fragile X mental retardation protein
GCL	ganglion cell layer
HBEGF	heparin-binding epidermal growth factor-like factor
HBP1	HMG-box transcription factor 1
HDAC9	histone deacetylase 9
HIF-1α	hypoxia-inducible factor-1α
HLF	hepatic leukemia factor
IE	inner ear
INL	inner nuclear layer
IPL	inner plexiform layer
IS	inner segment
ko	knockout
MIM	missing in metastasis
miRNA	microRNA
Mitf	microphthalmia-associated transcription factor
mRNA	messenger RNA
MTSS1	metastasis suppressor 1
NPC	neural progenitor cell
Nrf1	nuclear respiratory factor-1
nt	nucleotide
NV	neovascularization
OE	olfactory epithelia
ONL	outer nuclear layer
OPL	outer plexiform layer
OS	outer segment
P bodies	processing bodies
PDCD4	programmed cell death 4
Pdcd6	programmed cell death 6
PDGF-B	platelet-derived growth factor-B
PMF	primary myelofibrosis
polyQ	polyglutamine
pre-miR	miRNA precursor
pri-miR	primary miRNA transcript
Psen2	presenillin 2
qRT-PCR	quantitative reverse transcription-polymerase chain reaction
RASA1	RAS p21 protein activator 1
RB	retinoblastoma
Rdx	radixin
RISC	RNA-induced silencing complex
RNP	ribonucleoprotein
ROR	retinoic acid-related orphan receptor
RORA	retinoic acid receptor-related orphan receptor α
RORE	ROR elements
RP	retinitis pigmentosa
RPCs	retinal progenitor cells
RPE	retinal pigmented epithelium
RSCs	retinal stem cells
SCA	spinocerebellar ataxia
SCN	suprachiasmatic nuclei
siRNAs	small interfering RNAs
TMSB10	thymosin, beta 10
TOPORS	topoisomerase I-binding RS protein
TRBP	TAR RNA-binding protein
Ube2h	ubiquitin-conjugating enzyme E 2H
UTR	untranslated region
VEGF	vascular endothelial growth factor
VNO	vomeronasal organ
WS2	Waardenburg syndrome type 2
YWHAG	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma poly-peptide
ZT	Zeitgeber time

et al., 2006; Rehwinkel et al., 2005; Wu et al., 2006) and repress translation of the targeted mRNA by either blocking translation initiation (Chendrimada et al., 2007; Humphreys et al., 2005; Mathonnet et al., 2007; Pillai et al., 2005; Thermann and Hentze, 2007) and/or inhibiting elongation (Ambros, 2004; Lim et al., 2005; Maroney et al., 2006; Nottrott et al., 2006; Petersen et al., 2006; Zeng et al., 2003). Nucleotides 2–8 in the 5′ end of the mature miRNAs are recognized as "seed sequences", as they are the most important residues in determining the target sites (Bartel, 2004). Other features also influence the targeting specificity: for example, closely spaced target sites often enhance the effectiveness of each one of the sites; additional base pairing at nts 12–17 enhances miRNA targeting; AU-rich context flanking the target site favors the effectiveness of the targeting; and target sites positioning in the 3′UTR, away from the center of the long UTR and at least 15 nts from the stop codon, are preferred for an effective target site (Grimson et al., 2007). Although miRNAs seem to have repressive effects when their biding sites are artificially placed in the 5′UTR (Lytle et al., 2007) or coding regions (Kloosterman et al., 2004), whether miRNA targeting in the 5′UTR and coding regions occurs *in vivo* and confers physiological functions is still uncertain.

In addition to AGO proteins, other proteins in the RISC complex interact with AGO proteins and mediate inhibitory functions, e.g. GW182, *RCK/p54* helicase, the *Dcp1/Dcp2* decapping complex (Behm-Ansmant et al., 2006; Chu and Rana, 2006; Rehwinkel et al., 2005). miRNA-induced mRNA degradation and translation inhibition are considered to occur in the processing bodies (P bodies) in the cytoplasm, where miRNAs, the protein components of RISC, and the target mRNAs are enriched (Eulalio et al., 2007; Liu et al., 2005a,b; Minshall and Standart, 2004; Parker and Sheth, 2007; Sen and Blau, 2005; Smillie and Sommerville, 2002).

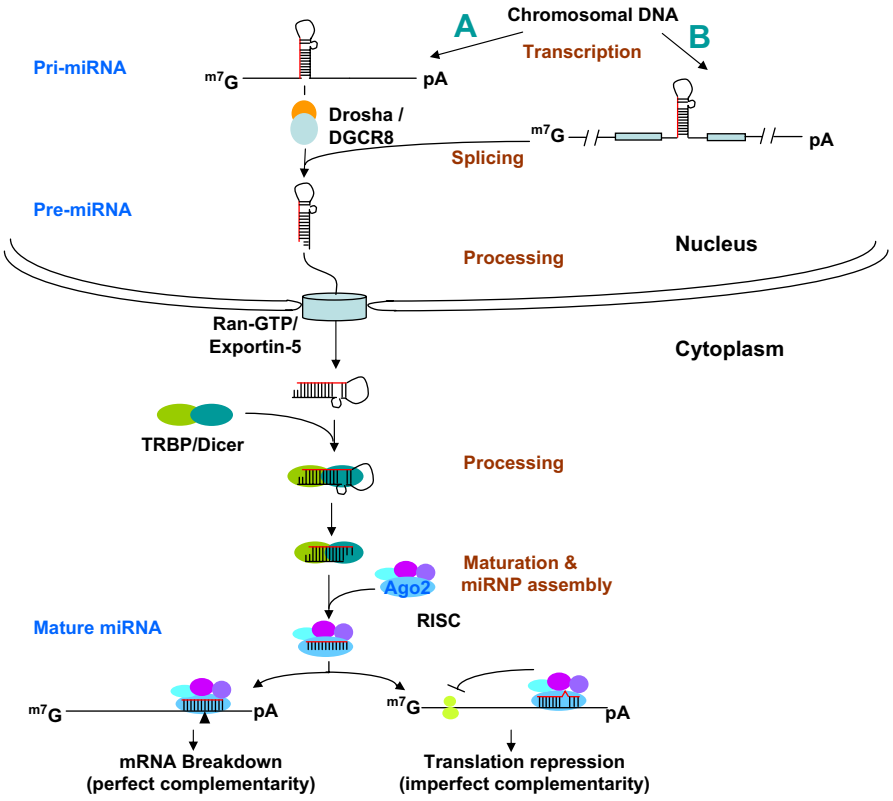


Fig. 1. miRNA biogenesis and functions. In the nucleus, the primary transcript of miRNA, referred as pri-miRNA, is transcribed by RNA polymerase II (A). Many miRNAs are derived from introns of protein-coding genes (B). The pri-miRNAs fold into hairpin structures, which are cleaved by an RNase III endonuclease, Drosha in the Drosha-DGCR8 complex, to form 60–70-nt stem loop intermediates, known as pre-miRNAs, with a 2-nt 3′ overhang. Pre-miRNAs are transported to the cytoplasm by Ran-GTP and an export receptor, Exportin 5. In the cytosol, pre-miRNAs are cleaved by another RNase III endonuclease, Dicer in the Dicer/TRBP complex, to form and ~22-bp miRNA duplexes with a 2-nt 3′ overhang. TRBP recruits Agonaute protein Ago2 and other Ago proteins to initiate the assembly of the RNA-induced silencing complex (RISC). One strand of the duplex becomes mature miRNA and is incorporated in the RISC complex; the other strand, miRNA*, is degraded. Mature miRNAs base pair with target mRNAs in the 3′UTR. When the miRNA and the target sites have precise or nearly precise complementarity, miRNAs specify cleavage of the target mRNAs by Ago2; when the base pairing is imperfect, miRNA with the RISC can destabilize the mRNA by deadenylation and subsequent decapping, and repress translation of the targeted mRNA by blocking translation initiation and/or inhibiting elongation.

Although most of the miRNAs are present in the cytoplasm regulating the abundance and translation of their target mRNAs, some miRNAs, e.g. *miR-29b*, are enriched in the nucleus, suggesting that these nucleus-enriched miRNAs also may regulate transcription or splicing of their target transcripts (Hwang et al., 2007). A hexanucleotide terminal motif of *miR-29b* appeared to act as a nuclear localization element, directing the enrichment of miRNAs and siRNAs in the nucleus (Hwang et al., 2007).

miRNAs comprise approximately 1–5% of animal genes (Bartel, 2004). It is estimated that there are more than 800–1000 miRNAs in the human genome (Bentwich et al., 2005). More than one-third of the protein-coding genes in the human genome are estimated to be subjected to miRNA regulation (Lewis et al., 2005; Xie et al., 2005). Each miRNA can target and regulate the mRNA transcripts of hundreds of downstream genes (Krek et al., 2005; Lewis et al., 2003; Lim et al., 2005). One miRNA can have multiple target sites in the mRNA transcript of a downstream gene, while one mRNA can be targeted by multiple miRNAs. Therefore, miRNAs contribute a newly recognized level of regulation of gene expression, playing important roles in the development and normal functions of almost all tissues and organ systems, such as the dorsal–ventral axis pattern formation (Martello et al., 2007), temporal pattern formation (Lee et al., 1993; Wightman et al., 1993), cell death and/or cell proliferation (Brennecke et al., 2003; Hipfner et al., 2002), fat storage (Xu et al., 2003), neuronal differentiation (Chang et al., 2004; Li and Carthew, 2005; Makeyev et al., 2007), muscle and heart development (Chen et al., 2006), hematopoietic lineage differentiation (Chen et al., 2004), and stem cell division and maintenance (Hatfield et al., 2005; Houbaviy et al., 2003). Defects in the biogenesis of miRNAs and mutations in miRNAs and/or the target sites in their target mRNAs can lead to abnormalities and diseases in animals and human (Abelson et al., 2005; Bernstein et al., 2003; Clop et al., 2006; Wienholds et al., 2003). miRNAs are shown to play important roles in tumorigenesis (Calin and Croce, 2006; Calin et al., 2004a,b; Croce, 2008; Esquela-Kerscher and Slack, 2006). Some miRNAs act as oncogenes (Eis et al., 2005; He et al., 2005; Huang et al., 2008; Ma et al., 2007) and some function as tumor suppressor genes (Calin et al., 2002; Cimmino et al., 2005; Johnson et al., 2005; Tavazoie et al., 2008). miRNA profiles of cancer tissues can be used to accurately identify their tissue origin and prognosis (Lu et al., 2005; Rosenfeld et al., 2008).

miRNAs, like small interfering RNAs (siRNAs), are small RNA molecules and are excellent targets for therapeutic drug development, once the roles of the miRNAs in the pathogenesis of human diseases are identified. Recently, novel classes of chemically engineered oligonucleotides, termed "antagomirs" or "antimiRs", have been developed and proved to be efficient and specific silencers of endogenous genes (Elmen et al., 2008a,b; Krutzfeldt et al., 2005; Stenvang et al., 2008). Therefore, a deeper understanding of the roles of miRNAs in the normal development and functions and their roles in the pathogenesis of diseases may facilitate the development of novel miRNA-based treatments of various diseases.

For more details on miRNA biogenesis, basic functions, and their roles in development and diseases in general, numerous excellent reviews are recommended: (Ambros, 2004; Bartel, 2004; Bushati and Cohen, 2007; Chang and Mendell, 2007; Filipowicz

et al., 2008; Kloosterman and Plasterk, 2006; Kosik, 2006; Stefani and Slack, 2008). The goal of this current review is to summarize recent advances and our current understanding of the roles of miRNAs in retinal and eye development, with emphasis on the retina. In the following chapters, I will first summarize the identification, characterization, and some functional studies of miRNAs in the retina and other ocular tissues in *Drosophila*, amphibians, zebrafish, and mammals, with an emphasis on the mammalian retina and eyes. Finally, I will focus on a highly conserved miRNA cluster – the *miR-183* cluster, which is highly enriched in the retina and other sensory organs and may play important roles in the development and functions of the retina and other sensory organs.

2. miRNAs in the eyes

In the past few years, several reports on miRNA expression in the retina and other ocular tissues, e.g. lens and cornea, in different species have started to uncover the miRNAs' involvement in the development and the functions of the retina and the eyes. These studies have paved the way for further investigation on the functions of miRNAs in the retina and other ocular tissues and related diseases.

2.1. *miRNAs in* Drosophila *eyes*

Although overall miRNA transcriptomes in *Drosophila* eyes have not been uncovered, numerous functional studies, focusing on different pathways, showed elegantly that miRNAs play important roles in cell proliferation, cell death, and cell-fate determination in *Drosophila* eyes.

Li et al. demonstrated that *miR-7* acts in a reciprocal negative feedback loop with a transcription factor, Yan, to promote photoreceptor cell fate, which is triggered by EGF receptor (EGFR) signaling (Li and Carthew, 2005) (Fig. 2). In *Drosophila* eye development, undifferentiated progenitor cells express Yan, an ETS-domain transcription factor, which acts as a repressor of retinal differentiation by transcriptional inhibition on its downstream target genes, including *miR-7*. Upon differentiation, EGFR signaling activates ERK-mediated phosphorylation of the Yan protein, resulting in rapid turnover of the Yan protein (Rebay and Rubin, 1995), releasing the repression of Yan on *miR-7* expression. *miR-7* normally starts to express in the differentiated photoreceptor cells when neuronal differentiation is first detected in the eye imaginal disc. In turn, *miR-7* further downregulates the expression of the Yan protein through its target sites in the Yan mRNA (Fig. 2). Therefore, *miR-7*, upregulated by EGFR signaling, works in concert with EGFR signaling to repress the Yan protein expression and promote photoreceptor differentiation (Li and Carthew, 2005).

Recently, *let-7* has been shown to be expressed in eye discs and involved in the normal development of *Drosophila* eyes, at least partially, through its regulation on *Drosophila dappled* (*DPLD*) (O'Farrell et al., 2008), a member of the Ring, B-box and coiled-coil/Tripartite Motif (RBCC/TRIM) superfamily. *DPLD* is expressed in developing and mature photoreceptors of *Drosophila* eyes and the optic lobe of the brain (O'Farrell et al., 2008). *DPLD* appears to be a homolog of *C. elegans Lin-41*, a heterochronic gene regulating the timing of cellular differentiation in *C. elegans* and one of the first genes whose transcript was demonstrated to be regulated by miRNA, *let-7* (Reinhart et al., 2000; Slack et al., 2000). As *lin-41*, *DPLD* was shown to be targeted and regulated by *let-7* in *Drosophila* eyes as well (O'Farrell et al., 2008), suggesting a conserved miRNA regulation on the *lin-41* homologs. Overexpression of *DPLD* under the control of GMR-Gal4, which drives the expression in all the cells behind the morphogenetic furrow (Freeman, 1996), causes a severe eye phenotype. The ommatidial units appear enlarged and many of the ommatidial units are fused. The lattice-like array organization of the ommatidia is disrupted and pigmentation is reduced, indicating the loss of pigment cells or dysfunction of these cells. This phenotype is sensitive to the dosage of *let-7*: deletion of one copy of the *let-7* gene enhances the eye phenotype, while duplication of the *let-7* locus appeared to mildly suppress the phenotype (O'Farrell et al., 2008), suggesting that *let-7* may regulate the expression of *DPLD in vivo* and play important roles in the development of *Drosophila* eyes.

Drosophila eyes have highly organized ommatidia. Changes in eye size, pigmentation, and ommatidial organization can be easily examined. Therefore, *Drosophila* eyes have been used as a model system of *in vivo* studies on neurodegenerative diseases (Bilen and Bonini, 2005) and studies dissecting miRNA biogenesis pathways (Bilen et al., 2006; Brennecke et al., 2003; Jin et al., 2004; Y. Lee et al., 2004; Y.S. Lee et al., 2004; Nolo et al., 2006; Thompson and Cohen, 2006).

Fragile X mental retardation protein (*FMRP*) is a selective RNA-binding protein. It interacts with miRNAs and RISC components, e.g. Dicer and *Ago1*, and plays important roles in the translational repression of miRNA-targeted mRNAs (Jin et al., 2004). Overexpression of *Drosophila dFMRP*, the orthologs of mammalian *FMRP*, in *Drosophila* eyes causes neuronal cell death and produces a consistent disruption of the ommatidia and mild rough eye phenotype (Wan et al., 2000). Loss of *AGO1* suppresses the rough eye phenotype caused by overexpression of *dFMRP*, suggesting that *dFMRP* interacts with *Ago1 in vivo* and is involved in miRNA-induced translational repression (Jin et al., 2004). *Me31B*, a DEAD-box RNA helicase, and its associated RNA-binding protein, *trailer hitch* (*tral*), are shown to interact with *dFMRP* in neuronal ribonucleoprotein (RNP) particles and be involved in FMRP-driven, argonaute-dependent, miRNA-mediated translational repression (Barbee et al., 2006). Loss of one copy of *Me31B* or *tral* suppresses *dFMRP* overexpression-induced retinal abnormality and rough eye phenotype (Barbee et al., 2006).

Drosophila miR-14 acts as a cell death inhibitor in *Drosophila* eyes by targeting *Drice*, an apoptotic effector caspase (Xu et al., 2003). Ectopic expression of *miR-14* in *Drosophila* eyes suppresses cell death induced by multiple stimuli, such as eye-specific expression of Hid, Grom, and Dronc, while loss of *miR-14* enhances Reaper-dependent cell death (Xu et al., 2003), suggesting that endogenous *miR-14* may modulate the Reaper-dependent apoptosis in developing eyes *in vivo*.

Misexpression of *Drosophila miR-278/mirvana* in developing eyes resulted in massive overgrowth and largely rescued the small and rough eye phenotypes induced by proapoptotic genes *Hid* and *Grim*, suggesting that *miR-278/mirvana* has strong anti-apoptotic activity (Nairz et al., 2006). The overgrowth appeared to be caused primarily by increased cell number (intervening cells and extra intraommatidial cells) in *Drosophila* eyes. Mutation in the seed sequence of mature *miR-278/mirvana* blocked the gain-of-function

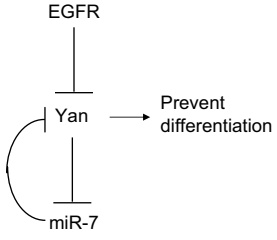


Fig. 2. In *Drosophila* eyes, EGFR signaling-induced upregulation of *miR-7* works in concert with EGFR signaling to repress the expression of Yan and promote photoreceptor differentiation (modified from Li and Carthew, 2005).

phenotype of massive overgrowth, but did not result in loss-of-function phenotype or enhance the severity of the small eye phenotype induced by Hid and Grim, suggesting that misexpressed miRNAs may acquire "novel" functions that, in this case, inhibit apoptosis. It exemplifies that amplification of certain miRNAs may potentially promote tumor formation (Nairz et al., 2006)

bantam locus in *Drosophila* was identified in a gain-of-function screening for genes that affect tissue growth. Overexpression of *bantam* by Gal4-dependent expression of EP elements inserted at the *bantam* locus causes tissue overgrowth, including the eyes. Flies homozygous for *bantam* deletion, $bantam^{\Delta 1}$, grow poorly and die as early pupae (Hipfner et al., 2002). The *bantam* gene encodes an miRNA, which simultaneously promotes cell proliferation and prevents apoptosis, at least in part by targeting and downregulation of the expression of the proapoptotic gene *Hid* (Brennecke et al., 2003). Recently, it has been shown that *bantam* is a target of Yorkie (*Yki*), which is the transcription activator in the Hippo tumor suppressor pathway in *Drosophila* (Edgar, 2006) and promotes growth by stimulating cell proliferation and inhibiting apoptosis (Nolo et al., 2006; Thompson and Cohen, 2006). *bantam* is required for *Yki*-induced overproliferation, and the growth of *yki* mutants can be fully rescued by overexpression of *bantam* (Nolo et al., 2006; Thompson and Cohen, 2006). In *Drosophila*, eye-specific expression of *Hid* with GMR-hid transgenes induces cell death in postmitotic cells in the eye imaginal disc (Bergmann et al., 1998; Kurada and White, 1998). Overexpression of *bantam* in the eyes of GMR-Hid flies largely restores eye size and ommatidial structures, while deletion of one copy of the endogenous *bantam* gene in GMR-hid transgenic flies enhances the severity of the GMR-Hid-induced small eye phenotype. This suggests that endogenous expression of the *bantam* gene in the developing eye may contribute to the control on the level of Hid-induced apoptosis, which is normally involved in reducing cell number in the pupal eye disc (Bergmann et al., 1998; Brennecke et al., 2003; Kurada and White, 1998). In *Drosophila* eye development, the ommatidia at the edge of the eye (rim cells) often lack a full complement of photoreceptors and support cells. They undergo apoptosis during mid-pupation to generate a smooth eye margin, which requires the expression of proapoptotic factors of Hid, Grim, and Reaper (Lin et al., 2004). Downregulation of *bantam* expression in these rim cells appears to be essential for the apoptosis of these cells because overexpression of *bantam* prevents the apoptosis of these rim cells (Thompson and Cohen, 2006).

With both pro-proliferation and anti-apoptotic functions, *bantam* also is shown to be a potent modulator suppressing the toxicity and neurodegeneration caused by polyglutamine (polyQ) expansion or abnormal accumulation of tau proteins (Bilen et al., 2006). Nine human neurodegenerative diseases are caused by expansion of CAG repeats, encoding glutamine, within the open reading frame of the respective genes. Spinocerebellar ataxia type 3 (SCA3) is one of the polyQ diseases with CAG expansion in the *Ataxin-3* gene (Zoghbi and Orr, 2000). Abnormal tau accumulation is associated with Alzheimer's disease and frontotemperal dementia (Lee et al., 2001). Expression of pathogenic ataxin-3 or tau in *Drosophila* eyes causes severe neurodegeneration and disruption of the pigmentation and/or retinal structures of the eye (Warrick et al., 1998; Wittmann et al., 2001). Blocking the miRNA-processing pathway dramatically enhances ataxin-3 and tau-induced neurodegeneration in *Drosophila* eyes, suggesting that the miRNA pathway modulates the toxicity of pathogenic polyQ and tau protein in different neurodegenerative diseases (Bilen et al., 2006). Interestingly, overexpression of *bantam* miRNA suppresses polyQ-induced neurodegeneration in the retina, while loss of function of *bantam* enhances the toxicity and neurodegeneration induced by polyQ ataxin-3, suggesting that endogenous *bantam* may help protect the eyes against polyQ-induced neurotoxicity (Bilen et al., 2006). This protective effect of *bantam* on polyQ toxicity appears to be downstream of the accumulation of the pathogenic proteins. Although it has been shown that *bantam* targets Hid and suppresses Hid-induced cell death (Brennecke et al., 2003), Hid-induced apoptosis and several other proteins of apoptotic pathways seem not to be involved in polyQ-induced neurodegeneration. The targets of *bantam* against polyQ- and tau-induced neurodegeneration may be distinct from Hid and several other genes that regulate apoptosis (Bilen et al., 2006).

Table 1
miRNAs expressed in zebrafish eyes.

Eye	Retina and localization of expression in the retina	Lens
miR-124a	All differentiated neurons (1, 2)	
miR-213	GCL (1)	
miR-9	CMZ maturing cells and mature amacrine cells (1, 2)	
miR-9*	Retina (1)	
miR-182	Rod/cone/bipolar, INL (1,2). Not as robust as miR-183 (2)	
miR-183	Rod/cone/bipolar, INL (1, 2). May have a peripheral-to-central gradient (2)	
miR-96	Rod/cone/bipolar, INL (1, 2). Not as robust as miR-183 (2)	
miR-184	RPE (3)	(+) (1, 3)
miR-204	RPE (1, 3)	(+) (1, 3)
miR-30a-5p	–	(+) (1)
miR-216	Retina (1)	
miR-217	Retina (1)	
miR-181a	Amacrine cells and GCL (1, 2)	
miR-181b	Amacrine cells and GCL and ciliary margin (1, 2, 3)	
miR-155	Retina (1)	
miR-19a	INL and GCL (3)	
miR-92b	CMZ (2,3)	
let-7b	CMZ (2)	
miR-454a	Retina (2)	
miR-132	CMZ, INL, GCL (low expressor) (2)	
miR-125b	CMZ, INL, GCL (low expressor) (2)	

References: (1) Wienholds et al. (2005); (2) Kapsimali et al. (2007); (3) Ason et al. (2006).

2.2. miRNAs in the zebrafish retina and eyes

In a systemic study of the spatial and temporal expression patterns of 115 conserved vertebrate miRNAs, Wienholds et al. reported that at least 15 miRNAs are expressed in the zebrafish retina (Table 1) (Wienholds et al., 2005). Kapsimali et al. performed *in situ* hybridization in the zebrafish brain and eyes and obtained detailed spatial expression profiles of 38 abundant, conserved miRNAs (Kapsimali et al., 2007), 12 of which are expressed in the retina (Table 1). In a study on the conservation of miRNA expression by comparison of the temporal and spatial expression patterns of miRNAs among zebrafish, medaka, chicken, and mouse, Ason et al. revealed more detailed expression patterns of some of the miRNAs expressed in the retina (Ason et al., 2006) (Table 1). In summary, at least 20 miRNAs have been confirmed to be expressed in zebrafish eyes (Table 1). Most of these miRNAs showed unique spatial and temporal patterns, suggesting that these miRNAs may have specific functions in different developmental stages and in different cell types.

miR-182, *miR-183*, and *miR-96*, which are members of a conserved sensory organ-enriched cluster, *miR-183/96/182* or the *miR-183* cluster (Xu et al., 2007b) (see below) are expressed in rods, cones, and bipolar cells in zebrafish, suggesting that they may play important roles in the photoreceptors and bipolar cells.

Both *miR-181a* and *miR-181b* are expressed in both amacrine and ganglion cells, while *miR-181b* also is detected in the ciliary margin zone (CMZ) (Ason et al., 2006; Kapsimali et al., 2007). *miR-9*

is expressed in mature amacrine cells, as well as in the CMZ (Kapsimali et al., 2007). *miR-213* is expressed only in the ganglion cells (Wienholds et al., 2005).

Some miRNAs are transiently expressed in the retina and may play some interesting roles in certain developmental stages. For example, *miR-92* is ubiquitously expressed and observed in the eyes, including the CMZ in the retina at early stages (before stage 34); however, in later developmental stages, the expression of *miR-92* in the eyes is turned off by stage 40 (Ason et al., 2006).

miR-124a is expressed in all differentiated neurons in the retina (Kapsimali et al., 2007), suggesting that *miR-124a* may play important roles in differentiation during development and normal functions of the adult retinas, while some other miRNAs are specifically expressed in proliferating progenitor cells and may play unique roles in regulating proliferation and/or differentiation during development, e.g. *miR-216* and *miR-217* are reported to be expressed in proliferative cells in the eyes (Wienholds et al., 2005).

In fish, retinal stem and progenitor cells (RSCs and RPCs) reside in the CMZ (also known as the circumferential germinal zone, CGZ) (Hitchcock et al., 2004). The RSCs and RPCs are aligned in the CMZ in a peripheral-to-central gradient, with the least committed stem cells in the most peripheral region (Harris and Perron, 1998; Hitchcock et al., 2004; Perron et al., 1998; Wehman et al., 2005). Progressive gene expression in the CMZ recapitulates the embryonic order of gene expression in the retina. More cell type-specification transcription factors are expressed, in gradient, from the peripheral to the central to direct retinal progenitor cells toward more committed cells and eventually postmitotic differentiated retinal neurons (Harris and Perron, 1998; Perron et al., 1998). miRNAs expressed in the CMZ may add another layer of the regulatory gradient modulating the proliferation and differentiation of RSCs and RPCs from the peripheral to the central regions of the CMZ. *let-7b* is specifically expressed in the peripheral region of the CMZ in the zebrafish retina (Kapsimali et al., 2007). Although only transiently expressed, *miR-92b* also is expressed in the CMZ before stage 34 during development (Ason et al., 2006). *miR-9* and *miR-181b* are expressed in the maturing cells in the CMZ, central to the *let-7* and *miR-92b* domains (Kapsimali et al., 2007). This unique spatial localization suggests that *let-7b* and *miR-92b* may be involved in the functions of RSCs or early RPCs, while *miR-9* and *miR-181b* may be involved in the specification of later progenitor cells and mature retinal neurons. Consistent with this hypothesis, in addition to the CMZ, *miR-181b* also is expressed in mature amacrine and ganglion cells (Ason et al., 2006; Kapsimali et al., 2007), and *miR-9* also is expressed in mature amacrine cells (Kapsimali et al., 2007). It is reasonable to speculate that miRNAs may have a unique signature in gradient in the CMZ to modulate the proliferation and differentiation of the RSCs and RPCs in concert with the transcription factors in the region. The data presented by Kapsimali et al. (2007) support this hypothesis. Identification of the miRNA transcriptomes in the CMZ and detailed studies on their temporal and spatial expression patterns in the CMZ will further strengthen this hypothesis and be of great importance in studying the roles of miRNAs in RSCs and RPCs. Such knowledge also may provide guidance on the roles of miRNAs in mammalian RSCs (Tropepe et al., 2000) and retinal development.

In zebrafish eyes, *miR-184* and *miR-204* are expressed in the retinal pigmented epithelia (RPE) and lens (Ason et al., 2006; Kapsimali et al., 2007), suggesting their potential roles in the RPE and lens. *miR-30a-5p* is detected in the lens (Kapsimali et al., 2007), suggesting that *miR-30a-5p* also may be involved in the development and functions of the lens in zebrafish.

Zebrafish has become a model system for genetic studies in the eyes (Fadool and Dowling, 2008). We expect that functional studies on miRNAs in zebrafish may soon elicit greater insight into the roles of miRNAs in retinal and ocular development and functions.

2.3. miRNAs in amphibian eyes

PA Tsonis group (Makarev et al., 2006) cloned small-sized RNA from newt eyes. By sequencing these clones, they confirmed that six of the miRNAs (*miR-124a*, *miR-125b*, *miR-181b*, *miR-133a*, *miR-21*, and *miR-7b*), shown to be expressed in zebrafish (Kapsimali et al., 2007; Wienholds et al., 2005) and mammalian eyes (Loscher et al., 2007; Ryan et al., 2006; Xu et al., 2007a,b), are expressed in newt eyes. Additionally, they reported 42 unknown candidate miRNAs. However, the identity of these candidate miRNAs still needs independent experimental confirmation, such as Northern blot and RT-PCR. Genomic localization and sequence analysis of the flanking sequences around these candidate miRNAs to determine whether the flanking sequences have the characteristics of pri-miRNAs, e.g. the hairpin structures, also may help confirm their identity as miRNAs in newt. The lack of newt genome sequences has hindered this process. Therefore, miRNA expression in newt eyes is still largely unknown. Although six miRNAs are confirmed to be expressed in the eyes, the spatial and temporal expression patterns and their functions in the eyes have not been studied.

The lens and retina of newt eyes have remarkable regenerative abilities (Del Rio-Tsonis and Tsonis, 2003), miRNA studies in newt eyes may be of special interest for studying miRNAs involved in the regeneration of the lens and the retina and may have important implications as to the functions of miRNA in the development of the lens, retina, and retinal stem cells in mammalian eyes.

2.4. miRNAs in mammalian eyes

2.4.1. miRNAs in the mammalian retina

2.4.1.1. Identification and characterization of miRNAs in the mammalian retina. In an original cloning and sequencing study on tissue-specific expression of miRNAs, Lagos-Quintana et al. (2003) first identified 21 miRNAs in adult mouse eyes. Nineteen of these 21 miRNAs are confirmed to be expressed in the adult mouse retina (Xu et al., 2007b).

Using miRNA microarray profiling, we identified that at least 78 miRNAs are expressed in the adult mouse retina (Supplemental Table 1 in Xu et al., 2007b). By comparison to the miRNA profiles of the adult mouse heart and brain, 21 of the 78 miRNAs appeared to be potentially retina-specific (Table 1 in Xu et al., 2007b). By quantitative (q)RT-PCR on a panel of RNA samples of 10 different adult tissues, six of the candidate miRNAs (*miR-96*, *miR-182*, *miR-183*, *miR-184*, *miR-210*, and *miR-140-AS*) were confirmed to be expressed only in the retina and not in the other nine tissues. Four of the 21 miRNAs (*miR-181c*, *miR-320*, *miR-31*, and *miR-211*) are preferentially expressed in the retina, while five others are preferentially expressed in the retina and brain (*miR-7*, *miR-9*, *miR-9-AS*, *miR-219*, and *miR-335*). These miRNAs are highly expressed in the adult retina and, therefore, may play important roles in normal functions of the retina. Forty of the 78 miRNAs expressed in the mouse retina also are expressed in both the brain and heart (Supplemental Table 8 in Xu et al., 2007b), suggesting that these miRNAs may have some "housekeeping" functions regulating the basic cellular functions in all tissues (Xu et al., 2007b). Although these miRNAs are expressed in all three tissues, many of them are expressed at significantly different levels (Supplemental Table 8 in Xu et al., 2007b). Since the mRNA transcriptomes in the three tissues are different, the downstream target genes for the same miRNAs may be different from tissue to tissue and, therefore, apparently commonly expressed miRNAs could have different functions in different tissues.

Ryan et al. (2006) reported 10 miRNAs expressed in the mouse retina, which are included in our adult retinal miRNA transcriptome (Xu et al., 2007b). Using miRNA microarray profiling, Loscher et al. (2007) also reported miRNA transcriptomes in the mouse retina,

which are consistent with our report (Xu et al., 2007b) with the addition of *miR-691* and *miR-26b* that are also preferentially expressed in the retina. Loscher et al. compared retinal miRNA expression profiles of two different mouse strains, c57 and 129, and showed only subtle differences between the two profiles: No deviations greater than twofold were detected and only 25 out of 640 probes on the microarray (Ambion) showed significant but smaller differences, suggesting that the tissue-specificity of the miRNA expression is highly conserved within the species (Loscher et al., 2007).

miRNA transcriptomes of the human neuroretina (our unpublished data) showed a very similar expression pattern when compared to miRNA transcriptomes in the adult mouse retina, suggesting that miRNA expression, and possibly their functions, are highly conserved between mouse and human.

With a different approach, Arora et al. selected 320 known "retinal genes" and performed target prediction on the 3′UTRs of these genes (Arora et al., 2007). Sixty-seven miRNAs are reported to have predicted target sites in the 3′UTRs of these "retinal genes". Using RT-PCR, 11 of these miRNAs (*miR-23a*, *miR-29*, *miR-107*, *miR-124*, *miR-7*, *let-7d* and *miR-135a*, *miR-135b*, *miR-143*, *miR-200b*, and *miR-206*) were confirmed to be expressed in human and rat retinas. The latter five were not detected in our microarray profiling results in adult mouse (Xu et al., 2007b) and human retinas (our unpublished data). They may be expressed at a very low level. Nevertheless, further confirmation may be needed to verify their expression in mammalian retina.

miRNA profiling in mouse and human retinas (Loscher et al., 2007; Ryan et al., 2006; Xu et al., 2007b) has greatly advanced our knowledge on miRNAs expressed in mammalian retinas; however, the spatial expression patterns of most miRNAs in the mammalian retina are still unknown. Only nine of the miRNAs expressed in adult mouse retinas have been localized by *in situ* hybridizations (Table 2). *miR-124a* is expressed in the newly differentiated neurons in developing retinas and in all mature neurons in adult retinas, but not in the Müller glial cells (Karali et al., 2007), consistent with the assertion that *miR-124* is expressed in differentiating and mature neurons, but not in neural precursors and astrocytes in the brain (Krichevsky et al., 2003; Makeyev et al., 2007; Miska et al., 2004; Smirnova et al., 2005). *miR-182*, *miR-183*, and *miR-96*, members of the *miR-183* cluster (see below), are highly expressed in the photoreceptors and interneurons in the inner nuclear layer (INL), but not in the RPE (Karali et al., 2007; Loscher et al., 2007; Ryan et al., 2006; Xu et al., 2007a,b). *miR-29c* is expressed in the photoreceptors and the outermost part of the INL, possibly in bipolar cells (Loscher et al., 2007). *miR-181a* is expressed in the ganglion cell layer (GCL) and the innermost part of the INL, possibly in amacrine cells (Karali et al., 2007; Loscher et al., 2007; Ryan et al., 2006). *let-7d* is shown to be expressed specifically in the INL, in both bipolar and amacrine cells (Loscher et al., 2007). *miR-9* is expressed in the middle part of the INL, possibly in Müller glial cells in the adult retina (Karali et al., 2007). *miR-204* is expressed in the RPE, GCL, and innermost part of the INL, possibly in amacrine cells (Karali et al., 2007). Interestingly, *miR-204* is also expressed in the ciliary epithelia (Karali et al., 2007; Ryan et al., 2006), where mammalian RSCs reside (Tropepe et al., 2000), suggesting potential involvement of *miR-204* in retinal stem cells as well as other functions of ciliary epithelia.

In spite of the limited data on spatial expression patterns, it appears that the expression patterns of most retinal miRNAs are conserved between zebrafish and mouse. For example, in both species, *miR-124a* is expressed in all differentiated retinal neurons; *miR-182*, *miR-183*, and *miR-96* are expressed in all photoreceptors and interneurons in the inner nuclear layer; and *miR-181* is expressed in both amacrine and ganglion cells. However, some differences exist between zebrafish and mouse. For example, *miR-204* is detected in the RPE and lens in zebrafish eyes (Ason et al., 2006), while in the mouse it also is expressed in amacrine and ganglion cells (Loscher et al., 2007), suggesting divergence of the expression regulation in different species.

2.4.1.2. miRNA expression in developing retinas in mouse. qRT-PCR analysis in retinal RNA samples of different developmental stages revealed that most adult retinal miRNAs have low or no expression in the early embryonic stages (Xu et al., 2007a,b), suggesting that different miRNAs may be expressed in various developmental stages in the retina to regulate the proliferation and sequential differentiation of RPCs.

To identify developmental stage-specific miRNAs, we isolated total RNA from eye cups at embryonic day (E) 10.5 and developing retinas at E14.5, E18.5, postnatal day 1 (P1) and P10 and performed miRNA profiling using miRNA microarrays (*mirVana* miRNA Bioarray-v2 (Ambion)). Our unpublished data showed that the number of miRNAs expressed at different developmental stages increases with development in the embryonic stages. P1 retinas have the highest number of miRNAs expressed. The number of miRNAs expressed in the postnatal retina declined slightly after P1. Approximately a third of the miRNAs expressed in the retina showed significant changes ($p < 0.001$) in their expression levels at different developmental stages. Most of these miRNAs showed increased levels of expression with development with peaks at postnatal stages. Some miRNAs have the highest expression at different embryonic stages; others are potentially expressed at only one developmental stage. These stage-specific or preferentially expressed miRNAs may play unique roles in the

Table 2
Localization of some retinal miRNA in mouse retina by in situ hybridization.

	Retina							Lens	Cornea
	RPE	ONL	INL-bipolar	INL-amacrine	INL-muller	GCL	CB		
miR-124a		+ (3)	+ (3)	+ (3)	− (3)	+ (3)			
miR-182	− (1–3)	+ (1–4)	+ (1–4)	+ (1–3)		+ (2)			
miR-183	− (1, 2)	+ (1, 2)	+ (1, 2)	+ (1, 2)		+ (3)			
miR-96	− (1)	+ (1)	+ (1)	+ (1)		+ (2–4)			
miR-204	+ (3)			+ (3)					
miR-9					+ (3)		+ (2, 3)		
miR-181a				+ (2–4)					
miR-29c		+ (3)	+ (3)						
let-7d			+ (4)	+ (4)					
miR-184								+ (3)	+ (2, 3)
miR-205									+ (2)
miR-217									+ (2)

References: (1) Xu et al. (2007a,b); (2) Ryan et al. (2006); (3) Karali et al. (2007); (4) Loscher et al. (2007).

fine-tuning of the proliferation/differentiation of RPCs and contribute to the tightly controlled spatial and temporal sequences in retinal development. Our ongoing confirmation and functional studies will further unveil their roles in retinal development.

2.4.1.3. miRNAs potentially involved in circadian clock and rhythmic functions of the retina. Circadian rhythms are daily cycles of physiology and behavior driven by an endogenous oscillator with a period of approximately 24 h (Aschoff, 1984; Bartel, 2004; Hastings et al., 2003; Pittendrigh, 1993). In mammals, the suprachiasmatic nuclei (SCN) of the hypothalamus function as the master circadian clock. SCN maintain their endogenous rhythm and synchronize the autonomous clocks in peripheral tissues, such as the retina, liver, kidney, and heart and, therefore, peripheral physiology and behavior (Hastings et al., 2003). At a molecular level, the core clock is composed of interlocking positive and negative transcriptional feedback loops that drive the rhythmic expression of the core clock components (Fig. 3). Transcriptional activators *Clock* and *Bmal1* heterodimerize and bind to E-box elements within the promoters of the *Period* (*Per*) and *Cryptochrome* (*Cry*) genes (Etchegaray et al., 2003; Gekakis et al., 1998; Hogenesch et al., 1998). *Per* and *Cry* proteins then associate with the *Clock*/*Bmal1* complex to repress their own expression, forming a 24-h feedback loop (Dunlap, 1999; Panda et al., 2002; Young and Kay, 2001). Additionally, the interplay between orphan nuclear receptors *Rev-erbα* and retinoic acid receptor-related orphan receptor α(*RORA*) maintains normal circadian expression of *Bmal1*: *Rev-erbα* functions as a transcriptional repressor of *Bmal1* expression, while *RORA* competes with *Rev-erbα* to bind to the ROR elements (RORE) in the promoter *Bmal1* gene and activates the expression of *Bmal1* (Preitner et al., 2002; Sato et al., 2004).

Mammalian retinas not only contain melanopsin-expressing, photosensitive ganglion cells (ipRGCs), which provide the light signal input to the SCN that is crucial for photic entrainment of the circadian clock (Berson et al., 2002; Dkhissi-Benyahya et al., 2007; Hattar et al., 2002, 2003, 2006), they also contain endogenous circadian pacemakers regulating the rhythmic functions of the retina (LaVail, 1976; Tosini and Menaker, 1996, 1998). Isolated retinas entrain to light and secrete melatonin rhythmically in constant dark (Tosini and Menaker, 1996). Circadian clocks in retinas, working in concert with the master pacemaker in SCN, control the rhythmic functions of the retina (Tosini and Menaker, 1996), e.g. rod outer segment disk shedding (Grace et al., 1996, 1999; LaVail, 1976; Terman et al., 1993), visual sensitivity (Bassi and Powers, 1986; Bassi and Powers, 1987; Walker and Olton, 1979), amplitudes of the waves in electroretinogram (ERG) (Barnard et al., 2006; Brandenburg et al., 1983; Manglapus et al., 1998; McGoogan and Cassone, 1999; Peters and Cassone, 2005; Ren and Li, 2004), extracellular pH (Dmitriev and Mangel, 2000, 2001), cGMP-gated channel sensitivity (Ko et al., 2001, 2003, 2004), and the number and morphology of synaptic ribbons (Balkema et al., 2001; Hull et al., 2006). These rhythms allow the retina to anticipate the more than 6-log unit change in illumination between day and night and function properly (Green and Besharse, 2004). Loss of synchronized retinal phagocytosis leads to age-related loss of photoreceptor function (Nandrot et al., 2004). The circadian rhythm of the retina influences susceptibility to light-induced damage (Organisciak et al., 2000; Sugawara et al., 1998; Wiechmann and O'Steen, 1992) and photoreceptor survival in animal models of retinal degeneration (Ogilvie and Speck, 2002).

The rhythmic functions are achieved by rhythmic expression of a wide variety of genes, e.g. melatonin (Li et al., 2005; Sakamoto et al., 2004a,b; Tosini and Menaker, 1996), dopamine (Doyle et al., 2002a,b), opsins (Korenbrot and Fernald, 1989; Li et al., 2005), and other phototransduction pathway components (Bowes et al., 1988; Brann and Cohen, 1987; McGinnis et al., 1992; Storch et al., 2007; Wiechmann and Sinacola, 1997). Transcriptional regulation by the clock genes has been shown to be one of the major mechanisms (Ueda et al., 2002, 2005) of the rhythmic expression of the genes. Post-translational modification also plays important regulatory roles (Busino et al., 2007; Gallego and Virshup, 2007; Gatfield and Schibler, 2007; Godinho et al., 2007; Lowrey et al., 2000; Siepka et al., 2007). miRNAs may add new levels of post-transcriptional regulation, modulating the clock and contributing to the rhythmic expression of their downstream genes. While each miRNA regulates hundreds of target mRNAs (Krek et al., 2005; Lewis et al., 2005), rhythmic expression of one miRNA may contribute to the

Table 3A
miRNAs upregulated at ZT17.

Name	Fold change (ZT17/ZT5)	Change *p* value
hsa_miR_106b	3.28	0.019
hsa_miR_30b	1.59	0.022
hsa_let_7f	1.58	0.0544
hsa_let_7i	1.50	0.040
hsa_miR_139	1.45	0.043
hsa_miR_126	1.39	0.093
hsa_miR_107	1.30	0.056
hsa_miR_182	1.25	0.068
hsa_miR_103	1.25	0.100
hsa_miR_124a	1.25	0.093

Table 3B
miRNAs downregulated at ZT17.

hsa_miR_422a	0.49	0.072
hsa_miR_422b	0.75	0.078

Reference: Xu et al. (2007b).

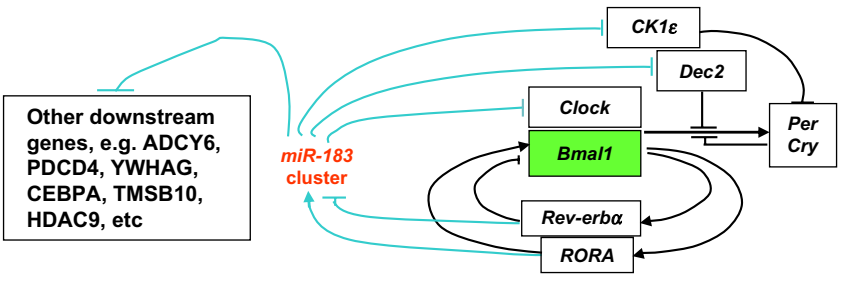


Fig. 3. Hypothetical involvement of members of the *miR-183* cluster in the circadian clock feedback loop (modified from Sato et al., 2004 and Xu et al., 2007b). The antagonistic regulation of *RORA* and *Reb-erbα* on the *miR-183* cluster gene may result in the rhythmic expression of the *miR-183* cluster; rhythmically expressed members of the *miR-183* cluster may regulate the expression of other clock genes, e.g. *clock*, *CKIε*, and *Dec2*, therefore, modulating the clock in the retina. Meanwhile, rhythmically expressed members of the *miR-183* cluster also may contribute to the rhythmic expression of many other downstream genes and the rhythmic function of the retina.

synchronization of the rhythmic expression of large numbers of genes, providing an efficient mechanism for rhythmic functions of the retina and other tissues.

By comparing the transcriptomes at 12:00 pm and 12:00 am, corresponding to Zeitgeber Time (ZT) 5 and ZT17, respectively, we identified 10 miRNAs whose expression levels were increased at ZT17, compared to those at ZT5 (Table 3A), while two miRNAs showed decreased levels at ZT17 (Table 3B) (Xu et al., 2007b). Using qRT-PCR, we confirmed the rhythmic expression of at least three of the 10 candidate miRNAs upregulated at ZT17: *miR-182*, *miR-126*, and *miR-139* (Fig. 4A, D and E) (Xu et al., 2007b). *miR-96*, another member of the *miR-183* cluster (see below and Xu et al., 2007b), also was tested and confirmed to have a similar rhythm to *miR-182* (Fig. 4B). Both *miR-182* and *miR-96* have the trough of expression around ZT5 and peak around ZT13 (Fig. 4A, B and Xu et al., 2007b) with peak/trough ratios of 1.7 and 2.7, respectively. *miR-126* and *miR-139* showed their peak expression in the later afternoon, around ZT9, and trough in the early morning around ZT21 and ZT1, with peak/trough ratios of 2.6 and 2.8, respectively (our unpublished data).

The rhythmic expression of these miRNAs may be regulated by core clock genes. These rhythmic miRNAs may target and modulate the expression of other clock genes and many other downstream genes, therefore contributing to the rhythmic expression of the downstream genes and, ultimately, the rhythmic function of the retina (Fig. 3 and see below).

2.4.1.4. Potential functions of miRNAs in retinal development and diseases

2.4.1.4.1. miRNAs may be involved in the normal laminar organization and functions of the retina. Dicer is one of the key enzymes to process pre-miRNAs into mature miRNAs during miRNA biogenesis. In a retina-specific Dicer conditional knockout (ko) model, *Chx10-cre*, *Dicer*^{flox}, Damiani et al. reported that levels of mature miRNAs were decreased in the ko retinas. Although the early development of the retina appeared "normal" and all the mature retinal cell types were detected by retinal cell type-specific antibodies, photoreceptor rosettes started to appear from P16 in the conditional ko animals, suggesting that miRNAs may be involved in the normal laminar organization of the retina. Following rosette formation, retinal degeneration occurs. ERG revealed decreased amplitudes of both *a* and *b* waves in both homozygous and heterozygous ko animals as early as one month of age (Damiani et al., 2008).

Since *Chx10-cre* expression is mosaic in the retina (Rowan and Cepko, 2004), *cre*-induced recombination does not occur in all progenitor cells and mature retinal bipolar cells. Therefore, Dicer was not inactivated in all progenitor cells in the developing retina in the *Chx10-cre*, *Dicer*$^{flox/flox}$ conditional ko mice. Because of this complication, many of the key issues could not be clearly addressed in this model. For example, although it appeared that all retinal cell types existed in the retina of the conditional ko mice, one cannot draw the conclusion that miRNAs are disposable for cell-fate determination in retinas because the mature retinal cells could be derived from RPCs, which escaped the *cre* excision and have intact Dicer and miRNA biogenesis. Although BrdU-labeling in the P35 retina and immunostaining using antibodies against phosphohistone H3 (PH3), a specific marker of cell proliferation, in the P16 retina did not detect cellular proliferation in the conditional ko animals, as in the wild type control animals (Damiani et al., 2008), the effect(s) of the ablation of miRNAs on the proliferation of RPCs are still unclear, since normally cellular proliferation stops around P10 in the mouse retina (Young, 1985a,b). Evaluation of BrdU-labeling and PH3-positive cells in different embryonic stages and

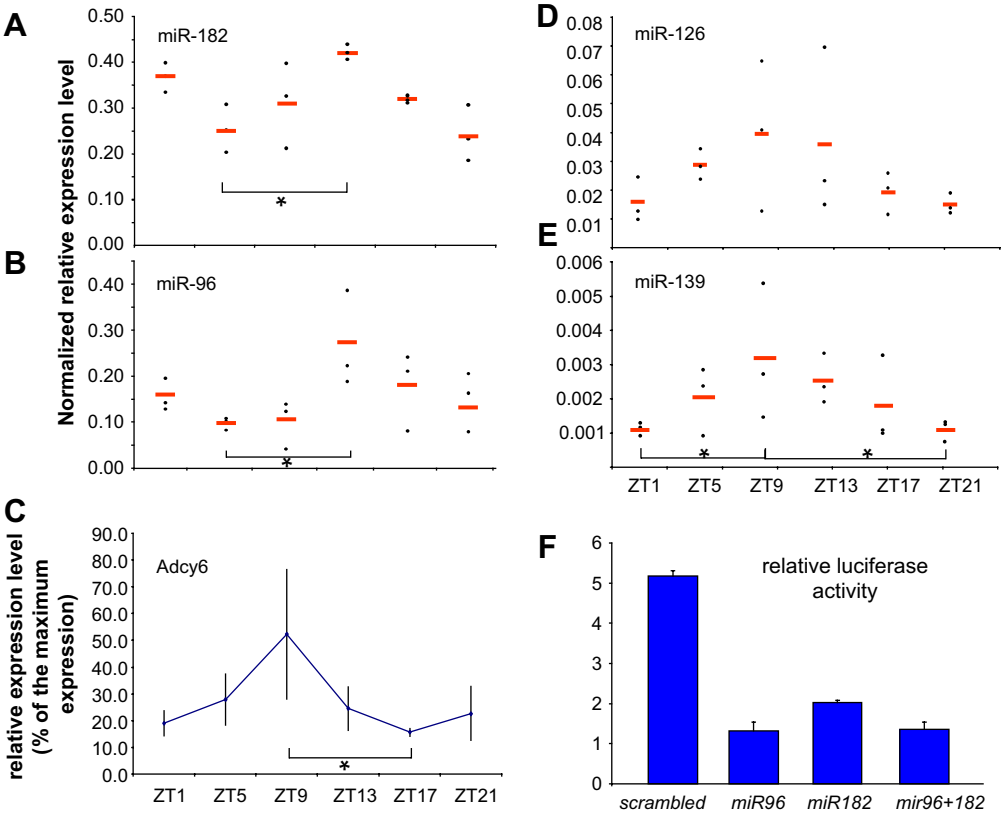


Fig. 4. (A)–(E) Relative expression levels of *miR-182*, *miR-96*, *Adcy6*, *miR-126*, and *miR-139* at ZT1, 5, 9, 13, 17, 21 ($n = 3$). The red bars indicate the mean value at each time point. $^{*}p < 0.05$ (Kruskal–Wallis test). (F) *miR*-96 and *miR-182* downregulate the expression of *Adcy6* in the luciferase reporter assay (Modified from Xu et al., 2007b).

early postnatal stages in Dicer ko RPCs in conditional ko animals is needed to further elucidate the roles of miRNAs in the proliferation of RPCs in the retina. The retinas of the *Chx10-cre*; *Dicer^{floxflox}* conditional ko mice undergo retinal degeneration, manifested by thinning of the outer nuclear layer and degeneration of the photoreceptor rosettes (Damiani et al., 2008); however, whether or not ablation of Dicer and miRNAs is directly responsible for cell death is unclear. The gradual cellular degeneration in the conditional ko retinas could be secondary to the disorganization of the retina and failure of the retinal neurons to establish proper synaptic connections. The fact that the early development of the retina appeared "normal" and the gradual degeneration of the retina occurred in later stages supports the latter hypothesis. A more conclusive answer will depend on direct examination of apoptosis in Dicer-inactivated cells in comparison to the Dicer-intact cells.

2.4.1.4.2. miRNAs involved in retinitis pigmentosa. To identify miRNAs involved in retinitis pigmentosa (RP), Loscher et al. (2007) compared retinal miRNA expression profiles of the wild type mice and Pro347Ser RHO transgenic mice. The Pro347Ser RHO transgenic mouse carries a transgene of a mutant form of rhodopsin with the dominant RP mutation, Pro347Ser (Li et al., 1996). In these animals, retinal degeneration occurs slower than most of the other RP mouse models, with little or no photoreceptor cell loss and roughly normal spatial expression of rhodopsin and ERG at age 1 month. However, rhodopsin-containing submicrometer-sized vesicles accumulate near the junctions between the inner and outer segments of the photoreceptors. Photoreceptors gradually degenerate, with up to 50% of the photoreceptors remaining at 4- to 5-month-old. Loscher et al. reported that expression of *miR-96* and *miR-183* was decreased by 2.5 fold, and *miR-1* and *miR-133* was increased by threefold in the retinas of 1-month-old Pro347Ser RHO transgenic mice when compared to the ones of wild type controls (Loscher et al., 2007), suggesting that these miRNAs may be involved in the progression of pathological changes in the retinas of this transgenic RP mouse model. *miR-1* and *miR-133* are both muscle-specific miRNAs (Lagos-Quintana et al., 2002) and are not normally expressed in the retina and neuronal tissues (Sempere et al., 2004; Xu et al., 2007b). They are co-transcribed as one pri-miRNA; however, mature *miR-1* and *miR-133* seem to have opposite effects on proliferation and maturation of muscle cells in both skeletal and heart muscles. Overexpression of *miR-133* causes increased proliferation and decreased myocyte differentiation (Chen et al., 2006), while ablation of *miR-1-2* results in the failure of cardiomyocyte to exit from cell cycles, leading to cardiac hyperplasia and prenatal or early postnatal death in approximately half the mutants (Zhao et al., 2007b). Loscher et al. analyzed potential targets of *miR-1* and *miR-133* and suggested that upregulation of *miR-1* and *miR-133* may repress the expression of an apoptosis inhibitor gene, Fas apoptotic inhibitor molecule (FAIM), and contributes to the apoptosis in the defective photoreceptors. Predicted targets of *miR-96* and *miR-183* also include apoptosis regulators, such as *Pdcd6* (programmed cell death 6) and *Psen2* (presenillin 2). Downregulation of *miR-96* and *miR-183* may result in de-repression of the expression of these genes. Therefore, both upregulation of *miR-1* and *miR-133* and downregulation of *miR-96* and *miR-183* may be involved in photoreceptor cell death in the pathogenesis of RP in the transgenic retina. Confirmation of the changes of the expression of these candidate downstream genes in the transgenic mouse retina will help support this hypothesis. Overexpression of *miR-1* and *miR-133* and knockdown of *miR-96* and *miR-183* in the retina may provide further insights into the functions of these changes of miRNAs in the progression of RP. *miR-183* and *miR-96* belong to a polycistronic miRNA cluster, the *miR-183* cluster, which includes *miR-182* as well as *miR-183* and *miR-96* (Xu et al., 2007b) (see below). Since they are co-transcribed as one pri-miRNA, *miR-182* also may be downregulated in the retinas of the Pro347Ser RHO transgenic mice. If so, the downstream target genes of *miR-182*, many of which overlap with *miR-96* and *miR-183* (Xu et al., 2007b), may be de-repressed and should be examined as well. This may provide more insight into the roles of miRNAs in the pathogenesis of RP in the transgenic retina. If these changes in miRNA expression truly contribute to the apoptosis in the Pro347Ser RHO transgenic retina, it is reasonable to speculate that similar changes of miRNA expression may occur in other forms of RP. If so, restoring the miRNA expression in the RP retina, e.g. using miRNA mimics of *miR-96* and *miR-183* and/or miRNA inhibitors of *miR-1* and *miR-133*, may have therapeutic effect(s) to delay programmed cell death in RP.

2.4.1.4.3. miRNAs involved in ocular neovascularization. Hypoxia-induced retinal and choroidal neovascularization (NV) are key pathological changes in various retinal diseases, e.g. diabetic retinopathy (Frank, 2004) and age-related macular degeneration (Green, 1999). miRNA profiling of ischemic retinas (from mice kept in 75% oxygen for five days) revealed that, under ischemic condition, seven miRNAs were upregulated, including *miR-451*, *-424*, *-146*, *-214*, *-199a*, *-181*, and *-106a*, when compared to control retinas, while three are downregulated, including *miR-31*, *-150*, and *miR-184* (Shen et al., 2008). Luciferase reporter assay and *in vivo* expression analysis revealed that *miR-31* may target and downregulate the expression of hypoxia-inducible factor-1α (*HIF-1α*) and platelet-derived growth factor-B (*PDGF-B*), while *miR-150* may target and downregulate the expression of *PDGF-B* and vascular endothelial growth factor (*VEGF*), a potent pro-angiogenic factor. Intraocular injection of *pre-miR-31* into ischemic eyes caused significant reductions in *PDGF-B*, *HIF-1α*, and *VEGF*. Injection of *pre-miR-150* also resulted in reduction in *PDGF-B* and *VEGF*, suggesting that downregulation of these miRNAs may contribute to ischemia-induced NV (Shen et al., 2008). Interestingly, *miR-31*, *-150* and *-184* also were downregulated in laser photocoagulation-induced choroidal NV. Although the target genes for *miR-184* were not confirmed, intraocular injection of *pre-miR-31*, *-184*, and *-150*, or a mixture of the three appeared to decrease retinal NV in ischemic retina and laser photocoagulation-induced choroidal NV (Shen et al., 2008), suggesting that these miRNAs may inhibit neovascularization *in vivo* and may potentially be used in the treatment of neovascularization in diabetic retinopathy (DR) and age-related macular degeneration (AMD).

2.4.2. miRNAs in other ocular tissues

2.4.2.1. Lens. miRNAs and miRNA-processing enzyme Dicer are expressed in the lens (Frederikse et al., 2006). In embryonic and early postnatal stages, Dicer protein is expressed in all differentiating fiber cells undergoing pronounced cell elongation and all epithelial cells, suggesting that miRNAs may be involved in the differentiation of the lens. In adult animals, Dicer protein is located to the outer cortex of the lens where the cells continue to differentiate throughout life, but not in the interior of the lens (Frederikse et al., 2006). By Northern blot, Frederikse et al. demonstrated that four miRNAs, *miR-124*, *miR-7*, *miR-125b*, and *let-7a*, were expressed, while muscle-specific *miR-1* was not expressed in the developing and adult mouse and rat lenses.

Using miRNA microarray profiling, Ryan et al. reported that at least 17 miRNAs were expressed in the adult mouse lens/ciliary body, including *miR-184*, *miR-125a* and *b*, *miR-31*, *miR-204*, *miR-26a* and *b*, *miR-181a* and *b*, *miR-30a*, *b*, *c* and *d*, *miR-23 a* and *b*, *miR-450*, and *let-7f* (Ryan et al., 2006). The expression of *miR-181*, *miR-184*, and *miR-204* was confirmed by Northern Blot. By *in situ* hybridization, Ryan et al. also demonstrated that *miR-184* is localized in the epithelia of the lens, with higher, uniform expression in the germinative region, but lower and punctated expression in the anterior region and that *miR-204* is uniformly expressed in the epithelia of the anterior region of the lens (Table 2).

Interestingly, most of the miRNAs highly expressed in the lens also are expressed in the retina (Ryan et al., 2006; Xu et al., 2007b) and the cornea (Ryan et al., 2006), including some miRNAs, which are highly, specifically expressed in the retina and cornea, e.g. *miR-184*, suggesting that ocular tissues may have common miRNA signatures.

miRNAs in the lens may be involved in the pathogenesis of cataracts. At least three miRNAs, *miR-31*, and *miR-99a* and *b* were reported to be downregulated after treatment of the rat lens and epithelial cells with cataractogens, e.g. ciglitazone (10 μM) or ZD2138 (100 μM) (Simic et al., 2008). By target prediction, Simic et al. suggested that *miR-31* may regulate cell cycle and proliferation of the lens' epithelial cells, while *miR-99a* and *b* may regulate the actin cytoskeleton and integrin-signaling pathway. However, the *in vivo* functional consequences of downregulation of these miRNAs in cataractogen-induced pathological changes and the pathogenesis of cataracts still need to be tested.

2.4.2.2. Cornea. By miRNA microarray profiling, Ryan et al. reported at least 31 miRNAs expressed in adult mouse corneas (Ryan et al., 2006). As in the lens, most of the highest expressed miRNAs (14/20 ~70%) in the cornea are expressed in the retina (Xu et al., 2007b), including *miR-184*, the highest expressed miRNA in the cornea (Ryan et al., 2006). By qRT-PCR, we showed that *miR-184* was specifically expressed in the retina, but not in 10 other tissues, including the brain, heart, liver, kidney, spleen, thymus, lung, testis, ovary (Xu et al., 2007b), and olfactory epithelia (unpublished data). Consistently, by multi-tissue Northern blot, Ryan et al. showed that *miR-184* is specifically expressed in the cornea, but not in nine other tissues, including footpad epithelia, tongue, small intestine, epidermis, and five of the tissues included in our study. Combining these data, *miR-184* appears to be exclusively expressed in ocular tissues, including the retina (Ryan et al., 2006; Xu et al., 2007b), cornea, and lens (Ryan et al., 2006). In the cornea, *miR-184* exhibits cell type-specific expression. It is localized primarily in the basal and immediately suprabasal cells of the corneal epithelium (Ryan et al., 2006) and endothelial cells (Karali et al., 2007), but not in the superficial cells of the cornea, the limbus, and conjunctival epithelia (Ryan et al., 2006). In contrast, *miR-205* and *miR-217* are expressed throughout the entire corneal, limbal, and conjunctival epithelia and mouse dermis. *miR-182*, a member of the *miR-183* cluster, appeared to have low expression in the corneal and limbal epithelia (Ryan et al., 2006). In a corneal epithelial regeneration test, it was shown that, 24 h after wounding to the corneal epithelia, *miR-184* was not detected in the newly formed regenerated corneal epithelia. However, it reappeared in the regenerated epithelia 48 hours after the wounding, suggesting that *miR-184* may be involved in the terminal differentiation of the corneal epithelia.

In summary, miRNA profiling in the retina and other ocular tissues has greatly increased our knowledge of the miRNAs expressed in the retina and eyes and opened a new field in retinal and eye research. However, the exact spatial expression patterns of most of these miRNAs are still unclear, and the *in vivo* functions of these miRNAs in retinal and ocular development are almost completely unknown. Investigation on the functions of miRNAs in the retina and other ocular tissues in the upcoming years may greatly enrich our knowledge of normal retinal and eye development and certain retinal and eye diseases.

Among the retinal miRNAs, we identified an miRNA cluster, the *miR-183* cluster, including *miR-182*, *miR-183*, and *miR-96*, which have showed unique expression patterns and a wide variety of potential functions in the retina and other sensory organs and in tumorigenesis. Although the *in vivo* functions of this miRNA cluster are still elusive, significant progress has been made to understand the expression regulation and other aspects of the potential functions of the *miR-183* cluster. In the following chapter, I will focus on studies of the *miR-183* cluster. Most of the research is still ongoing. This summary aims to review our current understanding and speculate on new directions of research.

3. Sensory organ-enriched miRNA cluster, the *miR-183* cluster

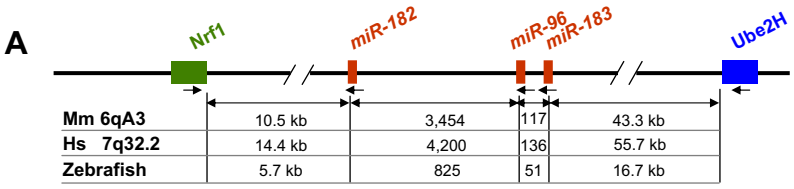
3.1. Identification and characterization of the miR-183 cluster as a sensory organ-enriched miRNA cluster

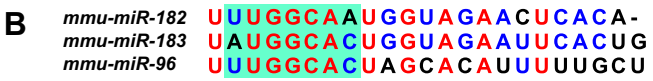
In our initial study on the miRNA transcriptomes of mouse retinas, we identified a sensory organ-enriched miRNA cluster, the *miR-183* cluster, including three of the highly expressed retinal miRNAs, *miR-183*, *miR-96*, and *miR-182* (Xu et al., 2007b). They are clustered within 4 kb on mouse chr6qA3, transcribed in the same direction (telomere → centromere), and located in an ~60 kb gap between the genes encoding nuclear respiratory factor-1 (*Nrf1*) and ubiquitin-conjugating enzyme E2H (*Ube2h*) (Fig. 5A). The orthologous region in humans is 7q32.2 where *hsa-miR-183*, *miR-96*, and *miR-182* have a similar arrangement (Fig. 5A). This genomic arrangement is conserved in the zebrafish (Fig. 5A). The sequences of these three miRNAs are similar to one another (Fig. 5B). All three of these miRNAs have similar developmental (Fig. 5C, D) and spatial expression patterns in the retina (Fig. 6B). RT-PCR of mouse retinal RNA showed that all three of these miRNAs are transcribed as a single polycistronic transcript (Xu et al., 2007b). This evidence suggests that these three miRNAs comprise a paralogous cluster. Therefore, we designated these three miRNAs as the *miR-183/96/182* cluster according to the transcription sequence (Xu et al., 2007b). To simplify the nomenclature, we refer to this cluster as the *miR-183* cluster. The expression of the *miR-183* cluster is not only restricted to the retina, but is highly expressed in all sensory organs as well, including mouse inner ear (IE) (Weston et al., 2006), olfactory epithelia (OE), the vomeronasal organ (VNO) (Choi et al., 2008; Xu et al., 2007b), dorsal root ganglia (DRG) (Kloosterman et al., 2006), and tongue epithelium (Xu et al., 2007b). Therefore, we consider the *miR-183* cluster as a unique sensory organ-enriched miRNA cluster.

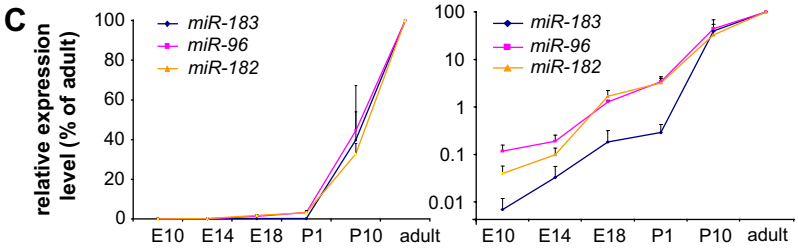
The tissue-specificity of the *miR-183* cluster appears to be highly conserved. Members of the *miR-183* cluster are expressed in all sensory organs of the zebrafish as well (Ason et al., 2006; Wienholds et al., 2005). Orthologs of *miR-183* exist throughout vertebrate and invertebrate deuterostomes, e.g. sea urchin and acorn worm, and protostomes, e.g. *Drosophila* and *C. elegans* that are separated by 600 million years of evolutionary divergence (Pierce et al., 2008). It appears that all *miR-183* family members are expressed in ciliated neurosensory organs in vertebrates and in innervated regions of invertebrate deuterostomes, suggesting that the *miR-183* cluster has ancient origins in evolution and may have crucial functions in neurosensory organ development.

3.2. Temporal and spatial expression patterns and expression regulation of miR-183 cluster

The *miR-183* cluster is an intergenic miRNA cluster, residing in the ~60 kb interval between *Nrf1* and *Ube2h* (Fig. 5A) (Xu et al., 2007b). Although the exact transcription start is unclear, promoter prediction analysis using PROSCAN (http://www-bimas.cit.nih.gov/molbio/proscan/) showed a putative promoter conserved in both human and mouse genomes, ~7 kb and 6 kb 5′ to *miR-183* in human and mouse, respectively (Fig. 6A and unpublished data). Sequence analysis of the genomic fragment upstream of *miR-183* revealed numerous predicted transcription factor-binding sites characteristic of genes expressed in neurosensory cells (Fig. 6A) (Xu et al., 2007b). These include at least one binding site for *CHX10*,



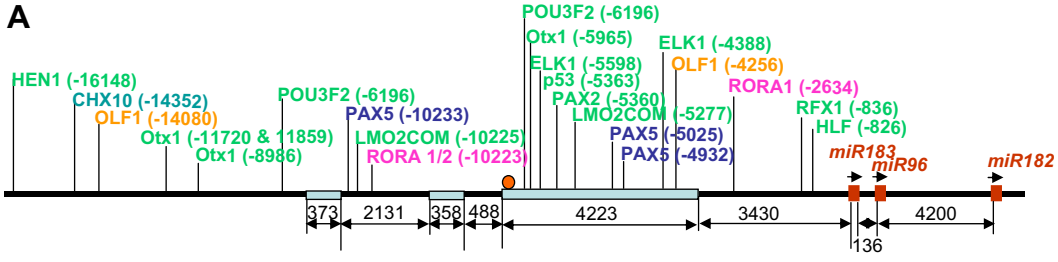




D **Analysis of Changes of The Expression of *miRs-96/182/183*.**

ratio	E14/E10	E18/E14	P1/E18	P10/P1	Adult/P10
miR-96	1.6	6.8	2.7	13.2	2.2
miR-182	2.4	17.2	1.9	10.2	3.1
miR-183	4.7	5.6	1.6	137.5	2.5

Fig. 5. Characterization of the *miR-183* cluster. (A) The genomic organization of the cluster is similar in mouse, human, and zebrafish. The numbers show the spacing in each of the three species in bp or kb. (B) Alignment of mature *miR-182*, *miR-96*, and *miR-183* sequences. Nucleotides in red are identical in all three; those in blue, identical in two of three. The aqua-shaded rectangle denotes the seed sequence. (C) Expression of *miR-182*, *miR-96*, and *miR-183* at different developmental stages. The relative expression level is represented as the percentage of the expression level in the adult retina. Results from three independent experiments were averaged and standard error of the mean (s.e.m) is presented as the error bar. The *Y*-axis of the graph on the right is in logarithm scale. (D) Quantitative analysis of the changes of the expression of members of the *miR-183* cluster at various developmental stages. (A)–(C) are modified from Xu et al. (2007b).



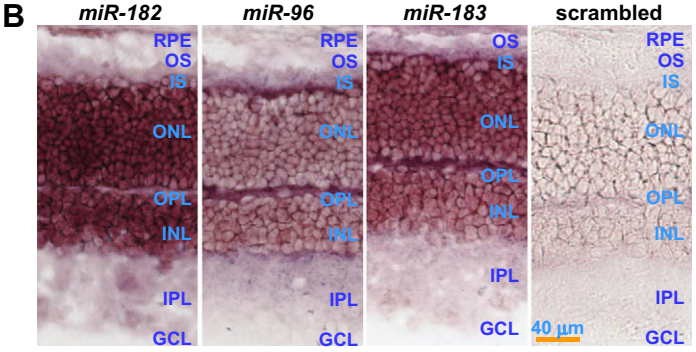


Fig. 6. (A) Potential 5′ upstream regulatory elements of the miRNA cluster on human Chr7q32.2 based on March 2006 Human Genome Assembly of UCSC Genome Informatics (http://genome.ucsc.edu). The color rectangles denote CpG islands; the putative transcription factor-binding sites are listed above. The numbers in parentheses are the location of the putative binding sites in reference to the first nucleotide of *pri-miR-183*; the numbers labeled below are the sizes of the fragments in base pairs. The orange dote represents the predicted promoter region. (B) *In situ* hybridization of *miR-182*, *miR-96*, and *miR-183* and a negative control probe in adult mouse retina. RPE: retinal pigment epithelium; OS: outer segment; IS: inner segment; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. (Modified from Xu et al., 2007b).

a key transcription factor in retinal development (Burmeister et al., 1996; Horsford et al., 2005; Liu et al., 1994), two binding sites for *OLF1*, an olfactory neuron-specific transcription factor (Wang et al., 1993), and multiple binding sites for *OTX1* (Fritzsch et al., 2001; Lanjuin et al., 2003; Martinez-Morales et al., 2001; Morsli et al., 1998, 1999) and *Pax2* (Baumer et al., 2003; Higashide et al., 2005; Kozmik et al., 2003; Schwarz et al., 2000; Torres et al., 1996; Yu et al., 2000), transcription factors important for the development of the retina and inner ear. Other predicted binding sites for sensory organ-related transcription factors in the 5′ region of the cluster also include *Pax5* (Kwak et al., 2006; O'Brien and Degnan, 2003; Wada et al., 1998), *Pou3F2* (Hagmann et al., 1995), *RFX1* (Dubruille et al., 2002; Ma et al., 2006; Swoboda et al., 2000; Vandaele et al., 2001), and *LMO2* (Deng et al., 2006). In different sensory organs, different combinations of transcription factors may contribute to the unique temporal and spatial expression patterns of the *miR-183* cluster.

In mice, it appears that the *miR-183* cluster is first expressed in the dorsal root and cranial ganglions, as early as embryonic day (E) 10.5, detected by *in situ* hybridization (Karali et al., 2007; Kloosterman et al., 2006). In the retina, the *miR-183* cluster is expressed at a very low level in the embryonic stages (Fig. 5C). Although their expression levels increase gradually in embryonic stages, by P1, their expressions are still ~1% or less of the adult expression level, and significant upregulation of their expression occurs only after birth, especially the stages from P1 to P10 (Fig. 5C, D) (Xu et al., 2007b). This developmental expression pattern coincides with the waves of differentiation of late-born retinal cell types (Cepko et al., 1996), including rod photoreceptors, bipolar cells, and Müller glia, suggesting that members of the *miR-183* cluster may be involved in the differentiation of late-born retinal cells and the functions of the adult retina.

Consistent with our quantitative RT-PCR result, members of the *miR-183* cluster were not detected on retinal sections of embryonic stages or at birth by *in situ* hybridization (Karali et al., 2007). The expression of members of the *miR-183* cluster in the retina was first detected by *in situ* hybridization at P8 (Karali et al., 2007). In adult retinas, the *miR-183* cluster appears to be expressed in the outer (photoreceptors, the primary sensory neurons) and inner nuclear layers (interneurons including bipolar, horizontal, and amacrine cells, the secondary neurons) (Fig. 6B) (Karali et al., 2007; Ryan et al., 2006; Xu et al., 2007b). Some evidence suggests that members of the *miR-183* cluster may be present in the outer segments of the photoreceptors and in their synapses to the bipolar cells (Karali et al., 2007), suggesting that members of the *miR-183* cluster may have specific local functions in the outer segments and the synapses of the photoreceptors.

Tissue-specific or cell type-specific and developmental stage-specific expression patterns of the *miR-183* cluster are possibly the results of the availability of various transcription factors and their concerted effects in specific tissues or cell types at specific developmental stages. Further dissection of the promoter region and other regulatory elements of the *miR-183* cluster, in correlation to the transcriptomes of specific tissues or cell types at specific developmental stages, will further deepen our understanding of the expression regulation of the *miR-183* cluster.

Forced expression of certain transcription factors, which may contribute to but may not be sufficient alone, for the expression of the *miR-183* cluster may result in ectopic expression. For example, *p53* may contribute to the expression of the *miR-183* cluster through a putative *p53*-binding site in the regulatory region of the *miR-183* cluster (Fig. 6A) (Xu et al., 2007b) (see below for more discussions on potential regulatory roles of *p53* on the *miR-183* cluster). The *miR-183* cluster is not normally expressed in the epithelia of the colon; however, activation of *p53* in colon cancer cell lines resulted in upregulation of *miR-182* (Chang et al., 2007).

Inactivation of key regulatory transcription factors may result in significant downregulation of the expression of the *miR-183* cluster. For example, there is at least one *Chx10*-binding site and two *RORA*-binding sites in the putative regulatory region of the *miR-183* cluster gene (Fig. 6A), suggesting that *Chx10* and *RORA* may contribute to the expression of the *miR-183* cluster in the retina. Consistent with this hypothesis, in the retinas of the *Chx10* mutant, $Chx10^{or\text{-}J/or\text{-}J}$, and *RORA* mutant, $RORA^{sg/sg}$, inactivation of *Chx10* or *RORA* resulted in significant downregulation of the expression of the *miR-183* cluster (our unpublished data and see below for further discussions).

There is also evidence suggesting that genomic amplification or deletion of the *miR-183* cluster locus in the genome could influence the expression level of member(s) of the *miR-183* cluster, especially in cancers (Calin et al., 2004a,b; Gaur et al., 2007; Zhang et al., 2006, 2008). Other epigenetic factors also are suggested to influence the expression of this cluster (Zhang et al., 2008).

Mature miRNAs are processed from primary transcripts, pri-miRs, through sequential cleavages by Drosha in the nucleus, and by Dicer in the cytoplasm. Therefore, the expression of mature miRNAs is subjected to post-transcriptional regulation in a tissue-specific and developmental stage-specific fashion (Mineno et al., 2006; Obernosterer et al., 2006; Thomson et al., 2006; Viswanathan et al., 2008; Wulczyn et al., 2007). Although members of the *miR-183* cluster are co-transcribed as one pri-miR, differences in post-transcriptional regulation and the stability of the mature miRNAs may result in different relative expression levels of the mature miRNAs of the members of the *miR-183* cluster in different tissues or at different developmental stages in the same tissue. In retinas, all three miRNAs increase with development (Fig. 5C, D, Table 4). However, mature *miR-182* is predominant at all time points. The ratios among the three mature miRNAs change with development (Table 4). The changes in the level of expression and the ratios among these three miRNAs may result in changes in overall functions of the *miR-183* cluster in retinal development and function at different developmental stages. The *miR-183* cluster is expressed in both the outer and the inner nuclear layer (Fig. 6B). However, the relative expression levels of the members of the *miR-183* cluster in different layers and cell types may have cell type-specific differences. Additionally, differences in their transcriptomes in different cell types may confer different target genes for each miRNA of the *miR-183* cluster. Therefore, the functions of each member of the *miR-183* cluster and the overall functions of the *miR-183* cluster in different cell types can be quite different.

3.3. Rhythmic expression of the miR-183 cluster in the retina

Members of the *miR-183* cluster are not only among the highest expressed miRNAs in retinas, but also follow a unique circadian rhythmic pattern with peak expression around ZT13 (~8 pm) and trough around ZT5 (~12 noon) (Xu et al., 2007b). This rhythmic

Table 4
Relative expression levels of mature *miRs-182/183/96* at different developmental stages.

	182	183	96	Ratio
Adult				
Noon	693.9	491.7	368.3	1:0.71:0.53
Midnight	869.6	583.0	416.9	1:0.67:0.48
P10	227.6	60.6	59.6	1:0.27:0.26
P1	59.0	21.5	19.1	1:0.36:0.32
E18	36.4	4.7	9.7	1:0.13:0.27
E14	51.7	10.2	8.7	1:0.20:0.17
E10	2.9	1.0	2.1	1:0.34:0.72

The data are based on miRNA microarray data (Xu et al., 2007b; and unpublished data).

expression of the *miR-183* cluster appears to be a conserved function. In the *Drosophila* head, the only two miRNAs with rhythmic expression, *dme-miR-263a* and *dme-miR-263b*, are homologs of members of the *miR-183* cluster (Pierce et al., 2008; Yang et al., 2008). Since the majority of the clocks of *Drosophila* heads are believed to reside in the compound eyes (Zeng et al., 1994), Yang et al. suggested that the rhythmic expression of *dme-miR-263a* and *dme-miR-263b* in the head of *Drosophila* may represent their expression pattern in the compound eyes (Yang et al., 2008). *dme-miR-263a* and *dme-miR-263b* are not only rhythmically expressed, but also exhibit similar phases of the rhythm as the *miR-183* cluster in mouse retinas, with peak levels around midnight (ZT/CT13 and ZT/CT19) and trough around noon (ZT/CT1-7). While *dme-miR-263a* and *dme-miR-263b* are rhythmically expressed in both light/dark and constant dark conditions in wild type flies, the rhythm is completely lost in the arrhythmic mutant, cyc^{01} (Yang et al., 2008), strongly suggesting that the rhythmic expression of *dme-miR-263a* and *dme-miR-263b* is a function of the clock. This conserved feature of rhythmic expression may indicate that the *miR-183* cluster plays important roles in the evolutionarily conserved rhythmic function of the retina.

As in *Drosophila* eyes, the rhythmic expression of the *miR-183* cluster in mammalian eyes may be a function of the circadian clock as well. As we examined the putative transcriptional regulatory element in the 5′ region of the *miR-183* cluster, we found several putative binding sites for transcription factors known to be important in the regulation of circadian rhythms (Fig. 6A). These include multiple binding sites for *RORA1/2*, which is a transcriptional activator for *Bmal1* expression in the SCN (Kamphuis et al., 2005; Sato et al., 2004) and at least two potential binding sites for *ELK1*. Phosphorylation of *ELK1* by extracellular signal-related kinase (*ERK*) is increased by photic stimulation and is involved in the regulation and photic-resetting of free-running circadian rhythms (Coogan and Piggins, 2003). There is also a predicted binding site for Hepatic Leukemia Factor (*HLF*), a member of the PAR bZip transcription factor family, that is expressed rhythmically in the liver and SCN and activates the expression of *Per1* (Mitsui et al., 2001).

As we discussed above, *RORA* is a member of the ROR family and a member of the core clock genes (Fig. 3). It binds to ROR elements (Sato et al., 2004) and functions as a transcriptional activator of *Bmal1* transcription in the SCN (Kamphuis et al., 2005; Sato et al., 2004). *RORA* is rhythmically expressed in the SCN and the retina, with peak around ZT8-12 and trough around ZT20 in the SCN (Ino, 2004; Sato et al., 2004; Steinmayr et al., 1998; Tosini et al., 2007a,b; Ueda et al., 2002). The peak time of *RORA* is hours ahead of the peak of the *miR-183* cluster, supporting the hypothesis that *RORA* may serve as a transcriptional activator of the *miR-182* cluster and contribute to the rhythmic expression of this cluster. If so, we predict that expression of the *miR-183* cluster in the retinas would be downregulated in the *RORA* null mutant mice, $RORA^{sg/sg}$, which carry a frameshift deletion in the *RORA* gene, causing a truncation of the protein prior to the ligand-binding domain, resulting in a null allele (Hamilton et al., 1996; Sidman et al., 1962). Loss of function of *RORA* in the SCN results in significant reduction of *Bmal1* expression, leading to defects in circadian locomotor activity consolidation with a shortened ambulatory locomotor activity period (Sato et al., 2004). Recently, we harvested retinal RNA from $RORA^{sg/sg}$ and wild type littermates and performed qRT-PCR. The results showed that all three members of the *miR-183* cluster were dramatically downregulated in $RORA^{sg/sg}$ retinas when compared to the wild type littermate controls (our unpublished data), strongly supporting our hypothesis that *RORA* regulates the expression of the *miR-183* cluster.

In the core clock network, *Rev-erbα*, another member of the ROR family, competes with *RORA* to bind to ROREs in the promoter region of *Bmal1* and represses the expression of *Bmal1* (Preitner et al., 2002; Sato et al., 2004). The opposing activities of *RORA* and *Rev-erb α* are required for the normal rhythmic expression of *Bmal1* and the consolidation of daily locomotor activity (Sato et al., 2004). *Rev-erbα* has been shown to be expressed in the retina (Ino, 2004; Kamphuis et al., 2005; Steinmayr et al., 1998; Tosini et al., 2007a,b). We predict that *Rev-erbα* may be involved in the regulation of the expression of the *miR-183* cluster as well, and that the rhythmic expression of the *miR-183* cluster in the retina may be the result of the counteraction between *RORA* and *Rev-erbα*. Our ongoing study on the expression of the *miR-183* cluster in circadian cycles in the $RORA^{sg/sg}$ and *Rev-erbα* knockout animals may provide further proof for these hypotheses.

3.4. Function prediction and functional studies on the miR-183 cluster

To begin to understand the functions of the *miR-183* cluster, we performed target prediction using four different programs: PicTar (http://pictar. bio.nyu.edu), TargetScan (http://www.targetscan.org), miRBase Targets (http://microrna.sanger.ac.uk/cgi-bin/targets), and Diana-MicroT (http://diana.pcbi.upenn.edu/cgi-bin/micro_t.cgi). These different algorithms produced similar lists of target genes. Each member of the *miR-183* cluster has hundreds of predicted target genes (Xu et al., 2007a,b). Since the three members of the *miR-183* cluster have considerable sequence similarity (Fig. 5B), the lists of predicted downstream targets showed significant overlapping. Genes playing important roles in various sensory organs are among the list, consistent with their high-level expression in sensory organs (Xu et al., 2007a,b). Functional annotation analysis on the target genes suggests wide variety of potential functions of the *miR-183* cluster.

3.4.1. Members of the miR-183 cluster may modulate the circadian clock and contribute to the rhythmic functions of the retina

The rhythmic expression of the *miR-183* cluster may be a function of the core circadian clock. Members of the *miR-183* cluster may, in turn, target some clock genes, as well as many other downstream target genes, to modulate the circadian clock and contribute to the rhythmic expression of a large number of downstream genes and the rhythmic function of the retina. As an example, we showed previously that adenylyl cyclase VI (*Adcy6*), which plays important roles in the regulation of arylalkylamine N-acetyltransferase (*AANAT*), one of the key enzymes in melatonin synthesis (Han et al., 2005), is a predicted target for *miR-182* and *miR-96* with at least six target sites for each miRNA (Xu et al., 2007b). We showed that *Adcy6* is expressed in the retina with circadian variation (Fig. 4C), a pattern nearly inverse to that of *miR-182* and *miR-96*, about 4 hours out of register (Fig. 4A, B). Our luciferase reporter assay showed that *miR-96* and/or *miR-182* downregulated luciferase activity through the 3′UTR region of *Adcy6* carrying the target sites (Fig. 4D), strongly supporting that *Adcy6* is a direct target of *miR-96* and *miR-182*. The delay of the appearance of the trough of expression of *Adcy6* in reference to the peak of the *miR-183* cluster expression may reflect the time required for the miRNA-mediated process to reduce the level of the targeted mRNA *in vivo*.

Further examination of the predicted targets showed that multiple clock genes and other rhythmic genes are potential downstream targets for members of the *miR-183* cluster (Table 5 and Fig. 3). *Clock* has at least one conserved predicted target site for both *miR-182* and *miR-96*, and casein kinase I epsilon (*CKIϵ*) has one conserved target site for *miR-182*. *CKIϵ* interacts with *Per* proteins and is important for the phosphorylation of *Per* proteins and their turnover. Mutations in the *CKIϵ* gene (*tau* allele) result in decreased enzyme activity and hypophosphorylation of *Per* proteins, leading

Table 5
Clock genes and other rhythmic genes that are predicted targets of members of the *miR-183* cluster.

Target gene	miRNA (no. of target sites)
(A) Core *Clock* genes	
Clock	miR-182 (1)/miR-96 (1)
CKIε	miR-182 (1)
Dec2	miR-182 (1)
(B) Other rhythmic genes	
PDCD4	miR-183 (1)
TOB1	miR-182 (1)
YWHAG	miR-182 (1)/miR-96 (1)
TMSB10	miR-182 (1)/miR-96 (1)
CEBPA	miR-182 (1)/miR-96 (1)

to a dramatically shortened period length of circadian rhythm in hamsters (Lowrey et al., 2000). Differentially expressed in chondrocytes protein 2 (*Dec2*) or basic helix-loop-helix domain containing, class B, 3 (*BHLHB3*), which, together with *Dec1*, regulates the circadian clock through repression of *Clock*/*Bmal1*-induced transactivation of *Per1* (Honma et al., 2002). *Dec2* has a conserved target site for *miR-182*. It has been reported that *Dec1* and *Dec2* expression peaks in the subjective day (Honma et al., 2002), when *miR-182* expression is low (Fig. 4A), supporting that *Dec2* may be a direct target of *miR-182*.

In addition to these clock genes, at least five other genes rhythmically expressed across multiple tissues, including the SCN, liver and heart (Sato et al., 2004), also are predicted targets of members of the *miR-183* cluster (Table 5). All five of these genes have been shown to be expressed in the retina by digital Northern data (http://cgap.nci.nih.gov/SAGE/AnatomicViewer). These include: programmed cell death 4 (*PDCD4*), whose expression peaks around ZT5.5 in the SCN (Sato et al., 2004), which has one potential target site for *miR-183*; transducer of ERBB2, 1 (*TOB1*), which has one target site for *miR-182*; tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide (*YWHAG*), thymosin, beta 10 (*TMSB10*) and CCAAT/enhancer-binding protein, alpha (*CEBPA*), each of which has one potential target site for *miR-182* and *miR-96*. *miR-182* also has one target site on transcript 3 of Histone deacetylase 9 (*HDAC9*), while *miR-96* has two target sites for transcripts 3 and 4 of *HDAC9*. *HDAC9* is shown to be rhythmic and peak around CT4.5 (Storch et al., 2007), about the time when *miR-182* and *miR-96* are at the trough of their expressions (Xu et al., 2007b). These observations suggest that the involvement of the *miR-183* cluster in circadian rhythm regulation in the retina is possibly far beyond its regulation on *Adcy6*. Our current hypothetical model on the roles of the *miR-183* cluster in circadian regulation in the retina (Fig. 3) includes: (1) the rhythmic expression of the *miR-183* cluster is a function of the clock in the retina. The antagonistic regulatory functions of *RORA* and *Reb-erbα* on the expression of the *miR-183* cluster may play the major role in setting up the rhythmic expression of the *miR-183* cluster; (2) rhythmically expressed members of the *miR-183* cluster may modulate the rhythmic expression of other clock genes, e.g. *clock*, *CKIϵ*, and *Dec2*, in the retina; and (3) rhythmically expressed members of the *miR-183* cluster may contribute to the rhythmic expression of many other downstream genes and the rhythmic function of the retina.

It is important to point out that whether these predicted target genes are subject to the regulation of members of the *miR-183* cluster needs independent experimental verifications. Luciferase reporter assays (Xu et al., 2007a,b) in cultured cells may provide support for the potential targeting. However, *in vivo* reporter assays may be crucial to prove the putative regulatory roles of members of the *miR-183* cluster on the expression of the predicted target genes.

Two different circadian clocks have been proposed in mammalian retinas. Some suggested that the circadian clock in the retina resides in the photoreceptors (Sakamoto et al., 2006; Tosini et al., 2007a,b), while others suggested that the clock of the retina is in the inner retina, possibly in the amacrine cells (Doyle et al., 2002a,b; Ruan et al., 2006; Sakamoto et al., 2004a,b). It is possible that the clocks exist in both layers, interacting with each other, to coordinate the rhythmic function of the retina. Therefore, the *miR-183* cluster may be rhythmically expressed in both the photoreceptor and inner nuclear layer. A detailed *in situ* hybridization on retinal sections from animals at different time points in a circadian cycle may help determine the location of the rhythmic expression of the *miR-183* cluster and lend further support for the location of the circadian clock in the retina.

Circadian rhythm is an evolutionarily conserved function and one of the basic biological features of animals. In addition to the retina, multiple sensory domains have been shown to have circadian rhythms in their functions and some also may contribute to the entrainment of the circadian rhythm of animals (Granados-Fuentes et al., 2004; Granados-Fuentes et al., 2006). Circadian rhythm in sensory organs may allow the coordination of the functions of all systems of our body to cope with rhythmic changes in the environment. The *miR-183* cluster is highly enriched in all sensory organs. As in the retina, the *miR-183* cluster may be expressed rhythmically in all sensory organs and contribute to the rhythmic functions of all sensory organs, playing important roles in the coordination of the rhythmic functions of different sensory organs. Our ongoing study may provide more evidence supporting this hypothesis. If so, the *miR-183* cluster may become the first example to be expressed rhythmically in all major sensory domains and coordinate the rhythmic functions of all major sensory organs.

To further predict the possible roles of miRNAs in the core clock, we analyzed all the core clock genes, including *Clock*, *Bmal1*, *Per1*, *Per2*, *Cry1*, *Cry2*, *RORA*, and *Rev-erbα* by both PicTar and TargetScan programs. The result showed that, except for *RORA* and *Rev-erbα*, all clock genes have potential target sites for one or more miRNAs expressed in the retina (Table 6), including some of the candidate rhythmic miRNAs detected in our initial screening of miRNAs with diurnal expression patterns (Tables 6, 3A and 3B). Some of these miRNAs have target sites on more than one clock gene (Table 6, given in bold), e.g. *miR-24* has potential target sites on both *Per1* and *Per2*, *miR-106b* has potential targets on both *Clock* and *Cry2*, and *miR-30b* has potential targets on both *Clock* and *Per2*. These observations suggest that miRNAs' involvement in the circadian clock may be well beyond the *miR-183* cluster. Many other miRNAs could be involved in the modulation of the circadian clock at multiple levels of the interlocking regulatory network and play important roles in circadian clock and rhythmic functions in retinas.

Table 6
Retinal miRNAs with predicted target sites on the mRNAs of core clock genes.

Clock genes	miRNAs
Clock	miR-9, 19, 15, 16, **30**, 195, 130, **106** (R), 96 (R), 182 (R) *miR-181a*, *181b*, *103* (*R*), *107* (*R*)
Per 1	**miR-24**, 29b, 29c
Per 2	**miR-24**, 25, **30a-5p**, **30c**, **30d**, **30e**, **30b** (R) *miR-21*
Cry 1	miR-361
Cry 2	miR-7, 24, let-7a/b/c/d/e/g, let-7f,i (R), **106b** (R)
Bmall	miR-27a, 27b, *miR-9**, 320,
RORA	None
Rev-erbα	None

Italic ones are predicted by only one of the programs of PicTar or TargetScan, but not both; (R): candidate rhythmic miRNA detected by comparative profiling in retina. Bold: miRNAs potentially targeting more than one clock genes; Underlined: confirmed rhythmic miRNAs.

miRNAs may add new layers to the post-transcriptional regulation of the rhythmic expression of some clock genes and many other downstream genes by breaking down their transcript mRNAs and/or inhibiting translation of their transcripts. Each miRNA can regulate hundreds of target mRNAs (Krek et al., 2005; Lewis et al., 2005). The rhythmic changes in miRNA expression could contribute to synchronized rhythmic expression for large numbers of genes. Therefore, miRNAs may provide an efficient and economic means translating the rhythm of the core clock to the rhythmic function of the tissue. These principles also may apply to the functions of miRNAs in circadian regulation in the SCN and other peripheral tissues.

3.4.2. Members of the miR-183 cluster may promote differentiation and influence cell-fate determination during retinal development

All three members of the *miR-183* cluster are expressed at very low levels during embryonic stages, and their expression is dramatically upregulated after birth, especially between P1 and P10 (Fig. 5C, D), coinciding with the waves of differentiation of late-born retinal cells, including rod photoreceptors, bipolar cells, and Müller glial cells (Cepko et al., 1996; Young, 1985a,b), suggesting that the *miR-183* cluster may promote differentiation and contribute to cell-fate determination, especially the late-retinal neurons. Examination of the predicted target genes of members of the *miR-183* cluster revealed that multiple genes important for the maintenance of neural progenitor identity are potentially targeted by members of the *miR-183* cluster, suggesting that the *miR-183* cluster may promote differentiation by downregulation of the progenitor genes. For example, *Numb*, a mammalian homolog of the *Drosophila numb*, has at least one potential target site for both *miR-183* and *miR-96* (PicTar). In *Drosophila*, *numb* is important for the asymmetric division by neural progenitor cells (NPCs) to generate sensory organs in the peripheral nervous system and neuronal diversity in the central nervous system (CNS) (Brewster and Bodmer, 1995; Frise et al., 1996; Rhyu et al., 1994; Roegiers et al., 2001). In mammals, asymmetric *Numb* distribution is critical for asymmetric cell division of cortical stem cells and neuroblasts and required for the maintenance of neural stem cells (NSCs) and NPCs (Petersen et al., 2002; Shen et al., 2002; Zhong et al., 1996; Zhong et al., 1997). In an asymmetric division, *Numb* appears to be inherited only by the apical daughter cell, which keeps the progenitor cell identity, while the other daughter cell differentiates. Similar effects also have been observed in the rat retina (Cayouette et al., 2001). *miR-183* and *miR-96* may downregulate *Numb* in the RPCs to promote differentiation in the retina.

Sox2, a member of the group B SRY-related HMG box (Sox) family, carries one potential target site for *miR-182*. In CNS, *Sox2* expression marks the proliferating neural progenitors and is downregulated in the ventricular zone concomitant with their differentiation (Ellis et al., 2004; Ferri et al., 2004; Tanaka et al., 2004; Taranova et al., 2006). It acts as a dose-dependent regulator of sensory neuron development in the retina (Taranova et al., 2006), inner ear (Kiernan et al., 2005; Taranova et al., 2006) and taste buds (Okubo et al., 2006). In developing retinas, *Sox2* is exclusively expressed in RPCs. Coincident with retinal cell differentiation, *Sox2* is downregulated such that its expression is mutually exclusive of differentiated retinal neurons (Taranova et al., 2006). Therefore, *miR-182* may potentially repress the expression of *Sox2* through the potential target site in the transcript of *Sox2* and promote differentiation during retinal development.

Pax6, a paired domain and homeodomain-containing transcription factor, is one of the earliest genes expressed in the eye field and a master control gene for retinal and eye development (Gehring, 1996; Ton et al., 1991; Walther and Gruss, 1991). *Pax6* is required for the proliferation of RSCs (Xu et al., 2007a) and RPCs (Marquardt et al., 2001; Philips et al., 2005). In the target prediction by TargetScan, *Pax6* also is predicted as a target of *miR-182* and *miR-96*. Therefore, members of the *miR-183* cluster may promote differentiation through downregulation of *Pax6* expression in RPCs.

Doublecortin (*DCX*) is a specific marker for neuronal precursors and young migrating neuronal cells during development and in the adult CNS and is downregulated during neuronal maturation (Brown et al., 2003; Couillard-Despres et al., 2005; Francis et al., 1999; Gleeson et al., 1999). In the retina, high levels of expression of *DCX* were observed from E18-E20, and the expression was downregulated after birth (E.J. Lee et al., 2003), coinciding with the upregulation of the *miR-183* cluster. In the 3′UTR of *DCX*, there are 3, 4, and 6 predicted target sites for *miR-183*, *miR-96* and *miR-182*, respectively, suggesting potential regulatory roles for members of the *miR-183* cluster on *DCX* expression.

Hes1, a homolog to *Drosophila hairy and enhancer of split*, is downstream of *Notch1* in the Notch signaling pathway (Jarriault et al., 1995). *Hes1* and other components of the Notch signaling pathway are expressed in NPCs in the forebrain (Sasai et al., 1992) and RPCs in the retina. They are downregulated in differentiating and mature neurons, which is critical for neurogenesis in the retina (Bao and Cepko, 1997; Tomita et al., 1996). Persistent expression of *Hes1* blocks neuronal and glial differentiation in the cerebral cortex (Ishibashi et al., 1994). At least one predicted target site for both *miR-182* and *miR-96* in the 3′UTR of *Hes1* mRNA has been identified in target prediction, suggesting potential roles of *miR-182* and *miR-96* regulating the expression of *Hes1* and differentiation of progenitor cells in the retina.

Functional annotation analysis using Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/ and Dennis et al., 2003) on the predicted targets of members of the *miR-183* cluster showed significant enrichment in cell cycle-related genes (61 counts in total 846 DAVID IDs, $p = 3.8E\text{-}4$) (Table 7). Many of these genes are important for progression of cell cycles (48 counts in a total of 846 DAVID IDs, $p = 6.0E\text{-}3$) (Table 8), e.g. *CDK5R1*/*p35* (Tsai et al., 1994), *CDK5R2*/*p39* (Tang et al., 1995), *cyclin D2* (Inaba et al., 1992), *cyclin J*, *checkpoint suppressor 1* (Scott and Plon, 2005), etc., suggesting that members of the *miR-183* cluster may inhibit cell-cycle progression through downregulation on these cell-cycle progression-promoting genes.

While slowing down proliferation, members of the *miR-183* cluster also may promote differentiation of RPCs through downregulation of the expression of genes playing important roles in retinal differentiation. For example, *Hes1* promotes the formation of Müller glia in postnatal RPCs (Furukawa et al., 1997). *Pax6* is required for the multipotency of RPCs; when *Pax6* is inactivated, all progenitor cells adopt amacrine cell identity in differentiating retinas (Marquardt et al., 2001; Xu et al., 2007b). Therefore, downregulation of *Hes1* and *Pax6* in RPCs by members of the *miR-183* cluster may not only promote progenitor cells toward differentiation, but also influence the cell fate during differentiation.

3.4.3. Members of the miR-183 cluster may mediate the functions of Chx10, including the inhibition on Mitf, therefore contributing to the maintenance of neuroretinal identity

Microphthalmia-associated transcription factor (*Mitf*) is a key transcription factor required for the acquisition and maintenance of RPE cell identity (Horsford et al., 2005). Mutations in *Mitf* result in lack of RPE cell differentiation and transdifferentiation of RPE to neuroretina (Hodgkinson et al., 1993). In retinal development, *Mitf* is initially expressed across the entire optic vesicle, including both presumptive neuroretina and RPE, and is subsequently downregulated in the presumptive neuroretina when *Chx10* starts to be expressed around E9.5 (Nguyen and Arnheiter, 2000). *Chx10*-dependent downregulation of *Mitf* expression is critical for the establishment and maintenance of the neuroretina (Horsford et al., 2005; Nguyen and Arnheiter, 2000). In *Chx10* null mutant mice,

Table 7
Cell cycle-related genes in the predicted targets of members of the *miR-183* cluster.

NM_003936	Cyclin-dependent kinase 5, regulatory subunit 2 (p39)
NM_014762	24-Dehydrocholesterol reductase
NM_133646	Sterile alpha motif and leucine zipper containing kinase azk
NM_003463	Protein tyrosine phosphatase type IV a, member 1
NM_018243	Septin 11
NM_021111	Reversion-inducing-cysteine-rich protein with kazal motifs
NM_000919, NM_138821, NM_138822, NM_138766	Peptidylglycine alpha-amidating monooxygenase
NM_006717	Spindlin
NM_007065	CDC37 cell division cycle 37 homolog (*S. cerevisiae*)
NM_004985, NM_033360	v-ha-ras Harvey rat sarcoma viral oncogene homolog
NM_003885	Cyclin-dependent kinase 5, regulatory subunit 1 (p35)
NM_019063	Echinoderm microtubule associated protein like 4
NM_005665	Ecotropic viral integration site 5
NM_002656	Pleiomorphic adenoma gene-like 1
NM_005938	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, *Drosophila*)
NM_002520, NM_199185	Nucleophosmin (nucleolar phosphoprotein b23, numatrin)
NM_012325	Microtubule-associated protein, rp/eb family, member 1
NM_004561	OVO-like 1 (*Drosophila*)
NM_173354	SNF1-like kinase
NM_018667	Sphingomyelin phosphodiesterase 3, neutral membrane (neutral sphingomyelinase ii)
NM_004040	RAS homolog gene family, member B
NM_000633	B-cell CLL/lymphoma 2
NM_006283	Transforming, acidic coiled-coil containing protein 1
NM_014456, NM_145341	Programmed cell death 4 (neoplastic transformation inhibitor)
NM_002715, NM_004156	Protein phosphatase 2 (formerly 2a), catalytic subunit, alpha isoform
NM_003137	SFRS protein kinase 1
NM_002755	Mitogen-activated protein kinase kinase 1
NM_001429	E1A binding protein p300
NM_000127	Exostoses (multiple) 1
NM_182966	Neural precursor cell expressed, developmentally downregulated 9
NM_014223	Nuclear transcription factor y, gamma
NM_001759	Cyclin D2
NM_002737	Protein kinase C, alpha
NM_002923	Regulator of G-protein signalling 2, 24 kda
NM_015158, NM_153186	Ankyrin repeat domain 15
NM_001105	Activin A receptor, type I
NM_181041	Polybromo 1
NM_006079	CBP/p300-interacting transactivator, with Glu/asp-rich carboxy-terminal domain, 2
NM_001951	E2F transcription factor 5, p130-binding
NM_019084	Cyclin J
NM_000875	Insulin-like growth factor 1 receptor
NM_012257	HMG-box transcription factor 1
NM_003478	Cullin 5
NM_001706, NM_138931	B-cell CLL/lymphoma 6 (zinc finger protein 51)
NM_133433	Nipped-B homolog (*Drosophila*)
NM_012197	Rab GTPase activating protein 1
NM_003029	SHC (SRC homology 2 domain containing) transforming protein 1
NM_000430	Platelet-activating factor acetylhydrolase, isoform ib, alpha subunit 45kda
NM_005197	Checkpoint suppressor 1
NM_014521	SH3-domain binding protein 4
NM_033116	NIMA (never in mitosis gene a)-related kinase 9
NM_004958	FK506 binding protein 12-rapamycin associated protein 1
NM_032552	NGAP-like protein
NM_052851, NM_178006, NM_178007	Start domain containing 13
NM_001788	Septin 7
NM_007375	TAR DNA-binding protein
NM_005375	v-myb Myeloblastosis viral oncogene homolog (avian)
NM_014751	Metastasis suppressor 1
NM_178423	Histone deacetylase 9
NM_002010	Fibroblast growth factor 9 (glia-activating factor)
NM_199129	Ubiquitin-conjugating enzyme E2 variant 1

n = 61 (7.2%), p = 3.8E-4.

Chx10$^{or\text{-}J/or\text{-}J}$, *Mitf* is misexpressed ectopically in the neuroretina, which fails to keep its identity and deflects toward an "RPE-like" identity (Horsford et al., 2005; Rowan et al., 2004). Although a *Chx10* consensus DNA-binding site was observed in the promoter region of the *Mitf* genes, direct transcriptional repression on the transcription of *Mitf* by *Chx10* was not observed (Horsford et al., 2005). Therefore, in spite of the mutually exclusive expression of *Chx10* and *Mitf*, the exact mechanisms on the suppression of *Mitf* expression by *Chx10* are not fully understood.

As we described above, there is at least one *Chx10*-binding site in the putative upstream regulatory region of the *miR-183* cluster (Fig. 6A), suggesting a potential regulatory role of *Chx10* on the expression of the *miR-183* cluster. If *Chx10* upregulates the expression of the *miR-183* cluster, we predicted that the expression of this cluster would be downregulated in the *Chx10* null mutant mice, *Chx10* $^{or\text{-}J/or\text{-}J}$. The *or*^{J} allele has a C to A transversion in exon 3 of the *Chx10* gene, which converts codon 176 of *Chx10* from tyrosine to a premature stop codon (Tyr176Stop), resulting in a truncation of the protein in the first half of the homeobox domain of the *Chx10* protein and a complete loss of function (Burmeister et al., 1996). To test this hypothesis, we isolated total retinal RNA from P10-14 *Chx10*$^{or\text{-}J/or\text{-}J}$ mice and their wild type

Table 8
Predicted target genes of members of the *miR-183* clusters that are related to cell-cycle progression.

NM_014223	Nuclear transcription factor y, gamma
NM_003936	Cyclin-dependent kinase 5, regulatory subunit 2 (p39)
NM_001759	cyclin d2
NM_014762	24-Dehydrocholesterol reductase
NM_002737	Protein kinase C, alpha
NM_133646	Sterile alpha motif and leucine zipper containing kinase azk
NM_021111	Reversion-inducing-cysteine-rich protein with kazal motifs
NM_000919, NM_138821, NM_138822, NM_138766	Peptidylglycine alpha-amidating monooxygenase
NM_001105	Activin A receptor, type I
NM_015158, NM_153186	Ankyrin repeat domain 15
NM_181041	Polybromo 1
NM_006079	cbp/p300-interacting transactivator, with glu/asp-rich carboxy-terminal domain, 2
NM_007065	CDC37 cell division cycle 37 homolog (*S. cerevisiae*)
NM_001951	E2F Transcription factor 5, p130-binding
NM_019084	Cyclin J
NM_004985, NM_033360	v-ha-ras Harvey rat sarcoma viral oncogene homolog
NM_000875	Insulin-like growth factor 1 receptor
NM_003885	Cyclin-dependent kinase 5, regulatory subunit 1 (p35)
NM_012257	HMG-box transcription factor 1
NM_003478	Cullin 5
NM_001706, NM_138931	B-cell CLL/lymphoma 6 (zinc finger protein 51)
NM_019063	Echinoderm microtubule associated protein like 4
NM_005938	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, *Drosophila*); translocated to, 7
NM_002656	Pleiomorphic adenoma gene-like 1
NM_002520, NM_199185	Nucleophosmin (nucleolar phosphoprotein b23, numatrin)
NM_012325	Microtubule-associated protein, rp/eb family, member 1
NM_003029	SHC (SRC homology 2 domain containing) transforming protein 1
NM_004561	OVO-like 1(*Drosophila*)
NM_000430	Platelet-activating factor acetylhydrolase, isoform ib, alpha subunit 45kda
NM_173354	SNF1-like kinase
NM_005197	Checkpoint suppressor 1
NM_004040	RAS homolog gene family, member b
NM_033116	NIMA (never in mitosis gene a)-related kinase 9
NM_004958	FK506 binding protein 12-rapamycin associated protein 1
NM_000633	B-cell CLL/lymphoma 2
NM_032552	NGAP-like protein
NM_052851, NM_178006, NM_178007	Start domain containing 13
NM_014456, NM_145341	Programmed cell death 4 (neoplastic transformation inhibitor)
NM_002715, NM_004156	Protein phosphatase 2 (formerly 2a), catalytic subunit, alpha isoform
NM_007375	TAR DNA-binding protein
NM_003137	SFRS protein kinase 1
NM_002755	Mitogen-activated protein kinase kinase 1
NM_178423	Histone deacetylase 9
NM_014751	Metastasis suppressor 1
NM_002010	Fibroblast growth factor 9 (glia-activating factor)
NM_199129	Ubiquitin-conjugating enzyme e2 variant 1
NM_000127	Exostoses (multiple) 1
NM_182966	Neural precursor cell expressed, developmentally downregulated 9

n = 48 (5.7%), p = 6.0E-3.

littermates and performed qRT-PCR. Our result showed that there is a significant decrease of expression of all three members of the *miR-183* cluster in the neuroretinas of the mutant eyes when compared to their wild type controls (our unpublished data), strongly supporting the hypothesis that *Chx10* regulates the expression of the *miR-183* cluster. Previously, we showed that *Mitf* is a downstream target of both *miR-182* and *miR-96* with at least four predicted target sites for both *miR-182* and *miR-96* in its 3′UTR region (Xu et al., 2007b). By luciferase reporter assay, we demonstrated that *miR-182* and *miR-96* can downregulate the expression of *Mitf* through these potential target sites for both *miR-96* and *miR-182* (Xu et al., 2007b). Therefore, our data support the hypothesis that the *miR-183* cluster is downstream of *Chx10* and may mediate the functions of *Chx10*, e.g. the repression on *Mitf* (Fig. 7). However, our analysis on the temporal expression patterns of members of the *miR-183* cluster in the retina showed that they are expressed at very low levels or not at all in early embryonic stages (Fig. 5C and D) when the neuroretina was established. Therefore, we predict that *miRs-182/96*-mediated suppression on *Mitf* may not be the major mechanism during establishment of the neuroretina in early retinal development. Other mechanisms may exist to downregulate *Mitf* expression in the neuroretinas. *miRs-182/96* may contribute to the maintenance of the neuroretina in late embryonic development and in postnatal mouse eyes.

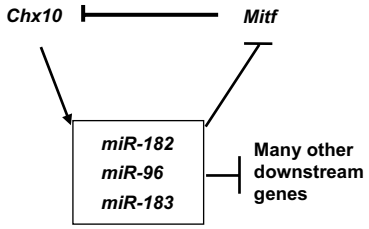


Fig. 7. The *miR-183* cluster may mediate *Chx10*'s repression on *Mitf*. *Chx10* may transactivate the expression of the *miR-183* cluster, members of which downregulate the expression of *Mitf*.

3.4.4. Potential roles of the miR-183 cluster in retinitis pigmentosa and other forms of retinal degeneration

As we discussed earlier, it was reported that expression of *miR-96* and *miR-183* was decreased by 2.5 fold in the retinas of 1-month-old Pro347Ser RHO transgenic mice when compared to wild type controls (Loscher et al., 2007), suggesting that these miRNAs may be involved in the progression of pathological changes in the retinas of this transgenic RP mouse model. Predicted targets of *miR-96* and *miR-183* include apoptosis regulators, such as *Pdcd6* and *Psen2*. Downregulation of *miR-96* and *miR-183* may result in de-repression of the expression of these genes and be involved in photoreceptor cell death and pathogenesis of RP in the transgenic retinas (Loscher et al., 2007).

Although no disease-causing mutations in miRNAs and in the target sites of their downstream genes have been reported to cause retinal and ocular diseases, misexpression of miRNAs has been reported to lead to disease in other systems (Yang et al., 2007). Mutations in the target sites in the transcripts of the downstream target genes of miRNAs also result in dysregulation of the target genes and lead to diseases in animals and humans (Abelson et al., 2005; Clop et al., 2006). Target prediction results showed that several genes responsible for retinitis pigmentosa when mutated are potential targets of members of the *miR-183* cluster; therefore, it is reasonable to speculate that mutations in members of the *miR-183* cluster or in the target sites of their downstream genes may result in dysregulation of these genes and directly contribute to RP or other retinal and ocular diseases. For example, mutations in the topoisomerase I-binding RS protein (*TOPORS*) gene cause autosomal dominant retinitis pigmentosa (adRP) (Chakarova et al., 2007) and account for about 1% of adRP (Bowne et al., 2008). TOPORS is a predicted target for both *miR-182* and *miR-96* with two potential target sites for each of these miRNAs. Therefore, misregulation of the *miR-183* cluster may potentially result in a change in expression level of TOPORS and possibly contribute to adRP.

As we discussed earlier, *SOX2* transcription factor is exclusively expressed in RPCs in developing retinas. Coincident with retinal differentiation, *Sox2* is downregulated such that its expression is almost mutually exclusive of differentiated retinal neurons (Taranova et al., 2006). Defects in *Sox2* cause microphthalmia syndrome type 3 (*MCOPS3*) (MIM:206900), including anophthalmia (Fantes et al., 2003), with optic nerve hypoplasia and abnormalities of the CNS (Kelberman et al., 2008), esophageal-genital syndrome (Williamson et al., 2006) and hearing loss (Hagstrom et al., 2005). *Sox2* is a predicted target of *miR-182* with one predicted target site in the 3′UTR region of its transcript. Changes in *miR-182* or its target site in *Sox* transcript may potentially cause changes in the expression of *Sox2* and may contribute to related eye and retinal phenotypes.

Many other predicted target genes of members of the *miR-183* cluster are known to be important for the development and normal functions of the retina (Xu et al., 2007b). Some have been discussed above, e.g. *Mitf*, *Pax6*, *Hes1*, etc. Mutations in these genes are known to cause severe retinal and ocular phenotypes (Hanson et al., 1994; Hodgkinson et al., 1993; Jordan et al., 1992; Lee et al., 2005; Steingrimsson et al., 1994; Xu et al., 2007b).

Theoretically, misexpression of members of the *miR-183* cluster and/or mutations in the target sites of members of the *miR-183* cluster may possibly lead to misregulation of these genes and cause or contribute to various retinal dysfunctions. Therefore, examination of the potential target sites for members of the *miR-183* cluster and other retinal miRNAs in known retinal disease-causing genes should be included in mutation screening studies on retinal and eye diseases in general, especially in the cases that mutations could not been found in the coding regions of the candidate, disease-causing genes linked to the phenotypes. Revisiting such cases with polymorphism analysis in the miRNA target sites in the 3′UTR regions may be fruitful to uncover potential miRNA-related retinal and eye diseases.

3.4.5. Potential roles of the miR-183 cluster in tumorigenesis

3.4.5.1. The miR-183 cluster may be downstream of p53 *and mediate some of the functions of* p53 *as a tumor suppressor gene.* Sequence analysis (Fig. 6A) revealed at least one conserved *p53*-binding site in the putative 5′ regulatory region of the *miR-183* cluster in both human and mouse genomes, suggesting potential role(s) of *p53* in the regulation of the *miR-183* cluster expression. Supporting this hypothesis, a recent report on colon cancer cell lines showed that, when the cells are treated with adriamycin, which activates *p53* and its downstream genes, expression of *miR-182* was significantly upregulated (Chang et al., 2007), suggesting that the *miR-183* cluster may be downstream of *p53* and that activation of *p53* may result in ectopic expression of the *miR-183* cluster.

In retinoblastoma (RB) tissues and RB cell lines, *p53* function is inactivated because of the amplification of the *p53* inhibitor, *MDMX* (Laurie et al., 2006). If the expression of the *miR-183* cluster is downstream of *p53*, we hypothesized that the *miR-183* cluster may be downregulated in the RB cell lines. To test this hypothesis, we prepared total RNA from two RB cell lines, Y79 and Weri1 (ATCC), and performed qRT-PCR. Our unpublished data showed that all three members of the *miR-183* cluster are not expressed or significantly downregulated in both RB cell lines, supporting the hypothesis that the *miR-183* cluster may be regulated *p53*.

If the *miR-183* cluster can be transactivated by *p53*, members of the *miR-183* cluster may mediate some of the functions of *p53* as a tumor suppressor gene. Functional annotation analysis using DAVID (http://david.abcc.ncifcrf.gov/ and Dennis et al., 2003) on the predicted target genes showed significant enrichment of proto-oncogenes (33 counts in a total of 846 DAVID IDs, $p = 1.3E\text{-}9$) (Table 9). These proto-oncogenes include the *pim-1* oncogene (Saris et al., 1991), rho family, small GTP-binding protein, *Rac1* (Didsbury et al., 1989; Polakis et al., 1989), *RAS p21* protein activator 1 (*RASA1*) (Cichowski et al., 1992), *KRAS2* (Deng et al., 1987), *MRAS* (Matsumoto et al., 1997), *RAB* 2C, 7,10, 21, 23, 34, 35, and 40B (Chen et al., 1993), *RHOB* (Madaule and Axel, 1985), *ret* proto-oncogene (Tahira et al., 1990), *v-yes-1* (Krueger et al., 1991), *v-crk* (ten Hoeve et al., 1993), *v-myb* (Janssen et al., 1986), and *ARHGEFs-3,4,6,7,12*, and *18* (Bagrodia et al., 1998), etc. Upregulation of the *miR-183* cluster by *p53* may potentially result in subsequent downregulation of these oncogenes.

Table 9
Proto-oncogenes that are predicted targets of members of the *miR-183* cluster.

NM_004349, NM_175634, NM_175635, NM_175636	Runt-related transcription factor 1; translocated to, 1 (cyclin d-related)
NM_020640	DCN1, defective in cullin neddylation 1, domain containing 1 (*S. cerevisiae*)
NM_002648	PIM-1 oncogene
NM_018243	Septin 11
NM_133646	Sterile alpha motif and leucine zipper containing kinase AZK
NM_175061	Juxtaposed with another zinc finger gene 1
NM_020630	RET proto-oncogene (multiple endocrine neoplasia and medullary thyroid carcinoma 1, Hirschsprung disease)
NM_194071	Camp responsive element binding protein 3-like 2
NM_004985, NM_033360	v-ha-ras Harvey rat sarcoma viral oncogene homolog
NM_004505	Hyperpolymorphic gene 1
NM_005402	v-ral Simian leukemia viral oncogene homolog A (RAS-related)
NM_001706, NM_138931	B-cell CLL/lymphoma 6 (zinc finger protein 51)
NM_153047, NM_153048	FYN oncogene related to SRC, FGR, YES
NM_001455, NM_201559	Forkhead box o3a
NM_005180	Polycomb group ring finger 4
NM_002655	Pleiomorphic adenoma gene 1
NM_005938	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, *Drosophila*); translocated to, 7
NM_002520, NM_199185	Nucleophosmin (nucleolar phosphoprotein b23, numatrin)
NM_015313	Rho guanine nucleotide exchange factor (GEF) 12
NM_004304	Anaplastic lymphoma kinase (ki-1)
NM_153649	Tropomyosin 3
NM_005433	v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1
NM_000633	B-cell CLL/lymphoma 2
NM_001731	B-cell translocation gene 1, anti-proliferative
NM_017778	Wolf–Hirschhorn syndrome candidate 1-like 1
NM_002015	Forkhead box o1a (rhabdomyosarcoma)
NM_152739	Homeobox a9
NM_003487, NM_139215	TAF15 RNA polymerase II, tata box binding protein (TBP)-associated factor, 68 kda
NM_006981, NM_173198, NM_173200, NM_006981	Nuclear receptor subfamily 4, group a, member 3
NM_005375	v-myb Myeloblastosis viral oncogene homolog (avian)
NM_006908, NM_018890, NM_198829	RAS-related c3 botulinum toxin substrate 1 (RHO family, small GTP-binding protein rac1)
NM_002890, NM_022650	RAS p21 protein activator (GTPase activating protein) 1
NM_006532	Elongation factor RNA polymerase II

$n = 33$ counts (3.9%), $p = 1.3E\text{-}9$.

As discussed above, cell cycle-related genes also are enriched in the predicted target genes (61 counts in a total of 846 DAVID IDs, $p = 3.8E\text{-}4$) (Table 7). Many of these genes are important for progression of cell cycles (48 counts in a total of 846 DAVID IDs, $p = 6.0E\text{-}3$) (Table 8). Therefore, members of the *miR-183* cluster may inhibit cell-cycle progression through downregulation on these cell-cycle progression-promoting genes. A significant enrichment in apoptosis-related genes also was observed in the predicted target genes (53 counts in a total of 846 DAVID IDs, $p = 1.2E\text{-}3$) (Table 10). Many of these apoptosis-related genes negatively regulate program cell death (20 counts in a total of 846

Table 10
Apoptosis-related genes that are predicted targets of members of the *miR-183* cluster.

NM_014762	24-Dehydrocholesterol reductase
NM_133646	Sterile alpha motif and leucine zipper containing kinase azk
NM_018434	Ring finger protein 130
NM_004985, NM_033360	v-ha-ras Harvey rat sarcoma viral oncogene homolog
NM_003885	Cyclin-dependent kinase 5, regulatory subunit 1 (p35)
NM_147156	Transmembrane protein 23
NM_001455, NM_201559	Forkhead box o3a
NM_033285	Tumor protein p53 inducible nuclear protein 1
NM_002656	Pleiomorphic adenoma gene-like 1
NM_002520, NM_199185	Nucleophosmin (nucleolar phosphoprotein b23, numatrin)
NM_004040	RAS homolog gene family, member B
NM_000633	B-cell CLL/lymphoma 2
NM_014800, NM_130442	Engulfment and cell motility 1
NM_005507	Cofilin 1 (non-muscle)
NM_199192, NM_199193	Brain and reproductive organ-expressed (TNFRSF1A modulator)
NM_003217	Testis enhanced gene transcript (BAX inhibitor 1)
NM_014456, NM_145341	Programmed cell death 4 (neoplastic transformation inhibitor)
NM_002715, NM_004156	Protein phosphatase 2 (formerly 2a), catalytic subunit, alpha isoform
NM_052842, NM_138639	BCL2-like 12 (proline rich)
NM_000966	Retinoic acid receptor, gamma
NM_004331	BCL2/adenovirus E1B 19 kda interacting protein 3-like
NM_001429	E1A binding protein p300
NM_004874	Silencer of death domains
NM_003374	Voltage-dependent anion channel 1
NM_004786	Thioredoxin-like 1
NM_002648	PIM-1 oncogene
NM_002737	Protein kinase C, alpha
NM_002575	Serpin peptidase inhibitor, clade b (ovalbumin), member 2
NM_001105	Activin A receptor, type I
NM_000875	Insulin-like growth factor 1 receptor
NM_004394	Death-associated protein
NM_080872	UNC-5 homolog D (*C. elegans*)
NM_002576	p21/Cdc42/Rac1-activated kinase 1 (STE20 homolog, yeast)
NM_005400	Protein kinase C, epsilon
NM_003478	Cullin 5
NM_001706, NM_138931	B-cell CLL/lymphoma 6 (zinc finger protein 51)
NM_020796	HT018 protein
NM_001224, NM_032982, NM_032983, NM_001224, NM_032983, NM_032982	Caspase 2, apoptosis-related cysteine peptidase (neural precursor cell expressed, developmentally downregulated 2)
NM_005802	Topoisomerase I-binding, arginine/serine-rich
NM_001380	Dedicator of cytokinesis 1
NM_014634	Protein phosphatase 1F (PP2C domain containing)
NM_005087	Fragile X mental retardation, autosomal homolog 1
NM_006377	UNC-13 homolog B (*C. elegans*)
NM_002657	Pleiomorphic adenoma gene-like 2
NM_001731	B-cell translocation gene 1, anti-proliferative
NM_002015	Forkhead box o1a (rhabdomyosarcoma)
NM_006570	RAS-related GTP-binding A
NM_144704	Apoptosis-inducing factor-like
NM_000447, NM_012486	Presenilin 2 (Alzheimer disease 4)
NM_002890, NM_022650	RAS p21 protein activator (GTPase activating protein) 1
NM_007008, NM_207521, NM_207520, NM_020532, NM_153828	Reticulon 4
NM_004309	Rho GDP dissociation inhibitor (GDI) alpha
NM_182757	IBR domain containing 2

$n = 53$ (6.3%), $p = 1.2E\text{-}3$.

Table 11
Negative regulators of apoptosis that are predicted targets of members of the *miR-183* cluster.

NM_014762	24-Dehydrocholesterol reductase
NM_002648	PIM-1 oncogene
NM_000633	B-cell CLL/lymphoma 2
NM_002575	Serpin peptidase inhibitor, clade b (ovalbumin), member 2
NM_001105	Activin A receptor, type I
NM_004985, NM_033360	v-ha-ras Harvey rat sarcoma viral oncogene homolog
NM_000875	Insulin-like growth factor 1 receptor
NM_005507	Cofilin 1 (non-muscle)
NM_199192, NM_199193	Brain and reproductive organ-expressed (tnfrsf1a modulator)
NM_003217	Testis enhanced gene transcript (BAX inhibitor 1)
NM_002015	Forkhead box o1a (rhabdomyosarcoma)
NM_147156	Transmembrane protein 23
NM_001706, NM_138931	B-cell CLL/lymphoma 6 (zinc finger protein 51)
NM_004331	BCL2/adenovirus E1B 19 kda interacting protein 3-like
NM_002890, NM_022650	RAS p21 protein activator (GTPase activating protein) 1
NM_002520, NM_199185	Nucleophosmin (nucleolar phosphoprotein b23, numatrin)
NM_007008, NM_207521, NM_207520, NM_020532, NM_153828	Reticulon 4
NM_001224, NM_032982, NM_032983, NM_001224, NM_032983, NM_032982	Caspase 2, apoptosis-related cysteine peptidase (neural precursor cell expressed, developmentally downregulated 2)
NM_004874	Silencer of death domains
NM_004309	Rho GDP dissociation inhibitor (GDI) alpha

$n = 20$ (2.4%), $p = 5.7E\text{-}3$.

DAVID IDs, $p = 5.7E\text{-}3$) (Table 11), e.g. B-cell CLL/lymphoma 2 (*BCL2*) (Borzillo et al., 1992), *BCL6* (Bray et al., 1991), Silencer of death domains or *BCL2*-associated athanogene 4 (*BAG4*) (Takayama et al., 1999), and 24-dehydrocholesterol reductase (*DHCR24*) (Di Stasi et al., 2005). Downregulation of these anti-apoptosis genes by members of the *miR-183* cluster may potentially promote apoptosis.

Several known metastasis-related genes are also among the targets of members of the *miR-183* cluster, e.g. Diphtheria toxin receptor (*DTR*) or heparin-binding epidermal growth factor-like factor (*HBEGF*) is predicted to be a common target for all three members of the *miR-183* cluster. *DTR* stimulates the growth of a variety of cells in an autocrine or paracrine manner, facilitates cell migration, and is a potent inducer of tumor growth and angiogenesis (Ongusaha et al., 2004). *DHCR24* is a predicted target gene of *miR-182* and *miR-96*. High levels of *DHCR24* expression are associated with melanoma metastases (Di Stasi et al., 2005). Members of the *miR-183* cluster may downregulate these genes and inhibit the metastasis of certain cancers.

However, it should be noted that potential functions of the *miR-183* cluster in modulation of cell cycle, apoptosis, and metastasis are probably more complicated than the predictions discussed above. Some of the predicted cell cycle-related target genes seem to cause cell-cycle arrest, e.g. sterile alpha motif and leucine zipper-containing kinase AZK (*ZAK*), which plays important roles in gamma radiation-induced cell-cycle arrest (Gross et al., 2002) and HMG-box transcription factor 1 (*HBP1*), which is shown to play a role in initiation and establishment of cell-cycle arrest during cellular differentiation (Shih et al., 1998). Some of the predicted apoptosis and metastasis-related genes are reported to promote apoptosis and inhibit metastasis. For example, *PDCD4*, a predicted target of *miR-183*, was shown to act as a tumor suppressor, inhibiting cell proliferation, promoting cell death, and inhibiting invasion and

metastasis (Goke et al., 2004; Leupold et al., 2007). Reversion-inducing-cysteine-rich protein with kazal motifs (*RECK*), a tumor and metastasis suppressor which negatively regulates matrix metalloproteinases and inhibits tumor metastasis and angiogenesis, is a predicted target of both *miR-182* and *miR-96* (Correa et al., 2006; Liu et al., 2003). A potential metastasis suppressor, Metastasis suppressor 1 (*MTSS1*) or Missing In Metastasis (*MIM*) (Lee et al., 2002), is predicted to be a common target for *miR-182* and *miR-96*. Downregulation of these targets, which potentially inhibit cell cycle and/or promote apoptosis and/or inhibit metastasis by members of the *miR-183* cluster, may potentially induce oncogenic effects.

These are seemingly contradictory to the analysis above that the *miR-183* cluster may act as a tumor suppressor downstream of *p53*. The roles of the *miR-183* cluster in tumorigenesis in different tissues may be different and dependent not only on the levels of miRNA expression, but also on the expression and levels of expression of different oncogenes and cell cycle-, apoptosis-, and metastasis-related genes, etc. The net effect of the two antagonizing sides of actions, oncogenic vs tumor suppressing, may eventually determine the roles of the *miR-183* cluster in tumorigenesis in different tissues, just like some of the classical proto-oncogenes which can have different functions to promote cell differentiation or proliferation depending on the cell types and act as tumor suppressors or oncogenes in different contexts (Zhang et al., 2001). For example, while activated *Kras2* promotes cancer development as a gain-of-function oncogene, wild type *Kras2* acts as a tumor suppressor, inhibiting carcinogenesis (Zhang et al., 2001). A single mutation in *Kras2* is usually not sufficient for neoplastic transformation and loss of the wild type allele is often required. It is tantalizing to consider that similar mechanisms may explain some of the seemingly conflicting predicted functions of members of the *miR-183* cluster: members of the wild type *miR-183* cluster may serve as tumor suppressors; however, mutations in member(s) of the *miR-183* cluster may abolish their tumor-suppressing functions and release their inhibition on many of the downstream oncogenic target genes, therefore manifesting a gain-of-function oncogenic effect. Mutations in members of the *miR-183* cluster also may alter their downstream target genes, resulting in unpredicted, new functions (gain-of-function). Loss of the wild type allele and/or amplification of the mutant allele may further contribute to oncogenesis. Human miRNA genes are frequently located at fragile sites and genomic regions involved in cancers (Calin et al., 2004a,b). *Chr7q32.2*, the genomic location of the *miR-183* cluster, has been recognized as one of the genomic fragile sites, FRA7H (Calin et al., 2004a,b) and possibly involved in chromosomal deletion in primary myelofibrosis (PMF) (Guglielmelli et al., 2007) or amplification in epithelial ovarian cancers (Zhang et al., 2008). Careful examination of the copy numbers of the *miR-183* cluster in the genome in combination with mutation screening in members of the *miR-183* cluster in cancer tissues may provide more evidence to test these hypotheses. Experimental tests on the actual effects of the *miR-183* cluster on cycle cycle regulation, cellular proliferation and apoptosis in different tissues and tumors with different tissue origins also will be critical to uncover the mysteries.

3.4.5.2. The miR-183 cluster in hematopoietic systems and leukemia. Originally, we identified the *miR-183* cluster as a sensory organ-*specific* miRNA cluster (Xu et al., 2007b). However, new emerging data have shown that members of the *miR-183* cluster also are expressed in bone marrow-derived CD34 + hematopoietic stem and/or progenitor cells (HSC–HPCs) (Georgantas et al., 2007) and normal B lymphocytes (Pal et al., 2007), suggesting that members of the *miR-183* cluster may be involved in hematopoiesis, especially in the differentiation of B-cell lineage. This is so far the only known tissue or cell types with *miR-183* cluster expression outside sensory organs. As we showed earlier, there are at least two *OLF1*-binding sites and three *Pax5*-binding sites in the regulatory region of the *miR-183* cluster gene (Fig. 6A). As a matter of fact, *Olf-1* was cloned and identified independently as early B-cell factor (*EBF*) (Hagman et al., 1993). *Pax5* also is known as B-cell-specific activator protein (*BSAP*). These transcription factors co-expressed in sensory organs and B-cell lineage, e.g. *OLF-1/EBF* and *Pax5*, may be responsible for the expression of this miRNA cluster in both systems.

miRNA profiling in different leukemia cell lines and hematopoietic cells from leukemia patients has produced an increasing body of provocative results, indicating that the *miR-183* cluster may be involved in leukemia and other types of cancers (Calin et al., 2004a,b; Guglielmelli et al., 2007; Navarro et al., 2008; Ongusaha et al., 2004; Pal et al., 2007), supporting the analysis on the potentially different roles of members of the *miR-183* cluster in tumorigenesis in different tissues discussed above. However, to keep the focus of the current review on the retina and the eyes, I will leave the details of the potential roles of the *miR-183* cluster in hematopoiesis and leukemia to a separate review.

3.4.6. Potential roles of the miR-183 cluster in other sensory organs and diseases

The *miR-183* cluster is highly enriched in the retina and other sensory organs. Increasing evidence suggests that the *miR-183* cluster may play important roles in the development and functions of multiple sensory organs. Mutations in members of the *miR-183* cluster may result in hearing loss and other sensory abnormalities (Lewis et al., 2007; Mencia et al., 2007; Modamio-Hoybjor et al., 2004; Xu et al., 2007a,b). However, to keep the focus of this current review, I will omit the details of the roles of the *miR-183* cluster in other sensory organs and leave it to a separate review as well.

3.4.7. Potential roles of the miR-183 cluster in autism

Autism is a heterogeneous syndrome defined by impairment in three core domains: social interaction, language, and stereotyped and repetitive behaviors (Abrahams and Geschwind, 2008; Kanner, 1943), affecting approximately 37 in 10,000 individuals (Abrahams and Geschwind, 2008). Sensory dysfunction has been noted in early studies on autism and is a common characteristic of autistic persons (Kanner, 1943; Kern, 2002; Kern et al., 2006), suggesting that a sensory processing problem may be considered part of the autistic disorder (Kern et al., 2007). Defects in multiple sensory modalities and multisensory processing in the brain may contribute to the development of autism. Multiple genome-wide linkage and association studies on autism provided increasing evidence for an autism susceptibility gene in 7q32.2, close to where the *miR-183* cluster gene is located (Barrett et al., 1999; IMGSAC, 1998; Lamb et al., 2005; Philippe et al., 1999; Risch et al., 1999; Schellenberg et al., 2006; Shao et al., 2002). More interestingly, multiple known autism-susceptibility genes (Abrahams and Geschwind, 2008) are predicted targets of members of the *miR-183* cluster (our unpublished data). The *miR-183* cluster is highly enriched in all sensory organs. Mutations in members of the *miR-183* cluster, and/or defects in the biogenesis of members of the *miR-183* cluster, may possibly result in dysfunction of multiple sensory modalities, which may contribute to defective sensory input to the brain, compromise information processing, and subsequent response to stimuli and appropriate social interaction and communication.

4. Conclusions and future directions

In the past few years, we and others have started to gain knowledge on miRNAs expressed in the retina, lens and cornea, especially in zebrafish (Wienholds et al., 2005) and mammalian

eyes (Loscher et al., 2007; Ryan et al., 2006; Xu et al., 2007a,b). However, this is only the start of an exciting new field, and major efforts are needed to unveil the functional roles of miRNAs in retinal and ocular development and functions.

Although the spatial expression patterns in the retina and eyes of about a dozen miRNAs have been localized by *in situ* hybridizations (Tables 1 and 2), the exact locations of the expression of most of the retinal and ocular miRNAs are still unknown. Retinal and ocular developments follow tightly controlled sequences spatially and temporally. Precise localization of the cell types expressing these miRNAs in the retina and ocular tissues, in relation to development will provide important clues to the potential functions of these miRNAs in the development of the retina and eyes.

miRNAs anneal to their target sites on the transcripts of their downstream genes by sequence complementarity and induce breakdown of the mRNAs and/or inhibition of translation from the transcripts. Different algorithms have been developed to predict potential target genes of miRNAs (PicTar, TargetScan, Miranda, miRBASE, DIANA-microT, etc). Using various functional annotation programs, e.g. DAVID and PathwayStudio, etc, one may predict potential functions of an miRNA based on the functions of its downstream target genes. However, the predicted interactions between the miRNA and many of its downstream targets may not occur *in vivo* in the tissue of interest, e.g. retina. Therefore, functional annotation analysis on the unsorted lists of predicted target genes cannot accurately predict tissue-specific functions of an miRNA. To date, mRNA transcriptomes of many tissues, including mouse and human retinas (Blackshaw et al., 2004; Blackshaw et al., 2003; Sharon et al., 2002), have been studied using either microarray hybridization or serial analysis of gene expression (SAGE) (Velculescu et al., 1995 and http://cgap.nci.nih.gov/SAGE/AnatomicViewer). We and others have screened the predicted target genes against the transcriptomes of the retina and selected target genes that are expressed in the retina (Arora et al., 2007; Loscher et al., 2007; Xu et al., 2007b) to make the functional prediction potentially more relevant to retinal biology.

However, the absence of expression of some of the predicted target genes of an miRNA in one tissue does not exclude the possibility that these genes may be targeted by the miRNA *in vivo*, since miRNAs tend to have mutually exclusive distribution with their downstream genes; when miRNAs and their downstream targets are co-expressed in a tissue, the downstream target genes tend to express at a low level when the miRNAs are highly expressed in the tissue (Farh et al., 2005; Sempere et al., 2004). Therefore, filtering the list of predicted target genes against the transcriptomes may miss some target genes, which may be endogenous targets *in vivo* but not expressed or expressed at a very low level in the tissue.

Therefore, biological experiments to directly detect the endogenous targets of the miRNAs are required to uncover their functions in the retina or other ocular tissues. Overexpression and/or knockdown of the expression of an miRNA, followed by comparative gene profiling, are two of the major approaches to experimentally determine the downstream targets of miRNAs. Overexpression of miRNAs can be achieved by transfection with miRNA mimics (Lim et al., 2005) or transduction with an miRNA-expressing virus, while expression of an miRNA can be knocked down by transfection with miRNA inhibitors, antimiRs (Elmen et al., 2008a,b), or antagomirs (Krutzfeldt et al., 2005). When comparing the transcriptomes of the cells or tissues with overexpression or knockdown of the miRNA(s) of interest, to the ones of the controls, the genes whose mRNA levels are downregulated or upregulated upon the overexpression or knockdown of the miRNA, respectively, are recognized as candidate target genes of the corresponding miRNA.

However, such experiments can only detect those targets whose mRNA levels are regulated by miRNA-mediated repression. The target genes, which are regulated by translation repression without changes in the levels of their transcripts, may not be detected by this approach. To detect target genes of the latter case, a genome-wide comparative proteomics may be the ultimate path to take. Recent advances in SILAC (stable-isotope labeling by amino acids in cultured cells) and pSILAC (pulsed SILAC) combined with mass spectrometry (Baek et al., 2008; Selbach et al., 2008) have provided a powerful means to quantify miRNA-induced translational repression and will greatly facilitate the identification of miRNA target gene.

The changes in the expression levels of the candidate target genes detected by comparative gene profiling or proteomics must be further confirmed by other independent methods, e.g. Northern blot, quantitative RT-PCR, or Western blot. miRNAs repress downstream genes by breaking down the mRNAs and/or inhibiting translation, thus the changes in protein level of a target gene may be more significant than the changes in mRNA levels (Selbach et al., 2008).

Whether a candidate target gene is regulated by the miRNA of interest can be further confirmed by luciferase reporter assays (Lewis et al., 2003; Xu et al., 2007b). To perform a luciferase reporter assay, first, the fragment of the 3′UTR of the candidate target gene carrying the potential target sites is subcloned into a luciferase reporter construct, 3′ to the luciferase cassette, e.g. renilla luciferase cassette, and 5′ to the polyA signal. Therefore, the subcloned 3′UTR fragment of the candidate target gene becomes the 3′UTR of the luciferase cassette in the reporter construct. Then the luciferase reporter construct is co-transfected with miRNA mimics duplex into cell lines that do not express the miRNA of interest. A control luciferase construct (e.g. firefly luciferase) is usually co-transfected for transfection control. Two to three days after transfection, luciferase activities are tested, and the *Renilla* luciferase activity is normalized by the firefly control luciferase activity. If the co-transfected miRNA downregulates the expression of luciferase through the potential target sites in the subcloned 3′UTR, the normalized luciferase activity may be decreased when compared to negative controls. *In vivo* targeting events can be monitored by *in vivo* transfection or by a transgene of an miRNA sensor, in which a luciferase or EGFP or β-galactosidase cassette with a 3′UTR fragment carrying the target sequences is introduced into a tissue by transfection or viral infection or into the animal through transgenic technology (Mansfield et al., 2004; Smirnova et al., 2005). If target sequences in the 3′UTR are functional and targeted by miRNAs *in vivo*, the luciferase activity or the intensity of the GFP or X-gal staining is expected to be decreased when the miRNA of interest is expressed endogenously in the tissue.

Creation of miRNA knockout (ko) and transgenic mouse models may further elucidate the function of retinal or ocular miRNAs *in vivo*. miRNAs play important roles in the fine-tuning of the gene expression and functions. Although complete ablation of miRNAs, e.g. by Dicer ko, leads to embryonic growth arrest and death (Bernstein et al., 2003; Wienholds et al., 2003), a classical ko of single tissue-specific miRNAs is less likely to result in embryonic lethal, therefore, more likely to produce ko models to study the *in vivo* roles of miRNAs in the development and functions of the retina and the eye. However, some miRNAs may function at different developmental stages with possibly different effects on retinal development. The effect of the miRNAs in a later stage may not be detected or compounded with the earlier effect of the miRNA(s) in traditional ko animals. Therefore, a conditional ko model using developmental stage- and cell type-specific promoter-driven Cre/loxP systems or inducible Cre/loxP systems may provide better models to study the effect(s) of miRNA(s) at specific developmental stages in specific cell types.

miRNAs function by sequence-specific targeting of the target sites in the 3′UTR of the transcripts of their downstream genes. Overexpression of miRNA(s) may disrupt the normal balance of gene expression and lead to certain phenotypes. Therefore, transgenic mouse models overexpressing miRNA(s) of interest in a retina- or eye-specific manner may be especially valuable for dissecting the functions of miRNAs in the retina and eyes when ko models could not produce detectable phenotypes. Mutations in mature miRNAs, especially in the seed sequences (Bartel, 2004), may not only decrease their binding affinity to their endogenous target genes (loss of function), but also may create new targets, hence new functions (gain-of-function), and result in unexpected phenotypes. Therefore, transgenics of the mutant miRNA also will be valuable to dissect the mechanisms of the disease or phenotype when mutant miRNAs are involved.

Misexpression of miRNAs can lead to disease (Yang et al., 2007). Mutations in the target sites in the transcripts of some downstream target genes of certain miRNAs also can result in misregulation of the target genes and lead to diseases in animals and humans (Abelson et al., 2005; Clop et al., 2006). Although no mutations in retinal and other ocular miRNAs and the target sites in their downstream target genes have been found to be directly responsible for retinal and eye diseases, we predict that, as in other tissues, mutations in miRNAs important for the development and functions of the retina and eyes may cause retinal malfunction and eye diseases yet to be discovered.

In summary, further investigation on the roles of miRNAs in retinal and ocular development and normal functions will not only greatly enrich our understanding of retinal and ocular biology, but also uncover miRNA-related molecular bases of retinal and ocular diseases and provide new strategies for the treatment of these diseases. Novel classes of chemically engineered oligonucleotides, termed ‘‘antagomirs’’ or ‘‘antimiRs’’, have been developed and proved to be efficient and specific silencers of endogenous genes (Elmen et al., 2008a,b; Krutzfeldt et al., 2005; Stenvang et al., 2008). miRNA-based novel treatment of retinal and ocular diseases may be developed once the roles of miRNAs in the pathogenesis of these diseases are identified.

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