Study of the role of miR-204 in photoreceptor development

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Study of the role of miR-204 in photoreceptor development

Thesis submitted for the Degree of Doctor of Philosophy

Life and Biomolecular Sciences

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ABSTRACT

MicroRNAs (miRNAs), a class of small non-coding RNAs with a basic role in post-transcriptional regulation of gene expression, are emerging as key players in the control of fundamental biological processes during the differentiation of many tissues and organs. The goal of my thesis is to shed light on the role of specific miRNAs in regulating the retina development especially photoreceptors. In particular, I focused on miR-204, one of the miRNAs our laboratory is mostly focusing on, and whose role in multiple aspects of eye development has been elucidated in the past few years from our lab and other groups. I dissected the role of miR-204 in photoreceptor cell differentiation and maturation by means of in vivo (Oryzias latipes i.e. medaka fish) and in vitro (661W cell line) systems. I found that, the gain-of-function of miR-204 led to an earlier exit of photoreceptor precursors from cell cycle and to a concomitant earlier ‘fate determination’, which in turn resulted in earlier onset of photoreceptor cell formation. Interestingly, I found that the induction of photoreceptor differentiation in 661W cells was accompanied by an up-regulation of miR-204 levels, supporting the hypothesis that miR-204 may play a role in cell cycle and/or cell fate determination. To better investigate on this aspect, I demonstrated that Cyclin D1 (Ccnd1) and Cd44 play a key role in miR-204-induced photoreceptor differentiation, as assessed by both in vivo and in vitro experiments. Moreover, I generated two medaka transgenic lines exclusively overexpressing miR-204 either in cone or in rod photoreceptors (TαC:GFP:miR-204 and Rho:TK:GFP:miR-204 respectively). Interestingly, I observed that cones and rods attained their maturation earlier respectively in the TαC:GFP:miR-204 and Rho:TK:GFP:miR-204 lines in comparison with controls. Overall this study strongly suggests a
prominent role of miR-204 in photoreceptor differentiation and maturation and sheds further light on the contribution of this miRNA to ocular development and function.
1. INTRODUCTION

1.1 The Retina

The eye is a complex, bilateral organ that develops through a highly ordered series of events during embryogenesis, which are conserved among different vertebrates. Proper establishment and function of the eye requires correct coordination and interaction of various tissues. The eye originates during the late gastrulation with the determination of the eye field in the anterior ectoderm. Later on, well-coordinated events lead to the formation of a single retinal primordium in the anterior neuroectoderm and the presumptive lens placodes in the adjacent head ectoderm (Jean et al., 1998). At late gastrula/early neural stage, the single retina field is split into two bilateral symmetrical retinal primordia by signalling mechanisms originating from the ventral midline (Gritsman et al., 1999; Li et al., 1997; Varga et al., 1999). Morphogenetic processes during neurula stages result in the evagination of the optic vesicle from the lateral wall of the forebrain. This process involves modulations in cell proliferation and apoptosis (Schmitt and Dowling, 1994). Simultaneously, lens specification happens when the optic vesicle comes in contact with the overlying ectoderm of the lens placode (Grainger, 1996). The optic vesicles invaginate under the influence of the developing lens to form the optic cup, where the differentiation of neural retina and retinal pigmented epithelium (RPE) occurs. The optic cup stays attached to the ventral diecencphalon with optic stalk, which assists as a path for the projection of retinal axons to reach their target regions in the brain (Bilitou and Ohnuma, 2010; Graw, 2003; Winkler et al., 2000) (Fig. 1.1).
The neural retina is a significant part of the eye, which senses light and sends information to the brain through the optic nerve. Notably, around 30% of the sensory input to the brain originates from retina, so the retina is referred as ‘‘window to the brain’’ (Swaroop et al., 2010). The vertebrate retina is composed of six different types of neuron cells and one type of glia organized in three nuclear layers. The Outer nuclear layer (ONL) is the outermost layer of the retina and contains the nuclei of rod and cone photoreceptors which detect the light stimuli or photons and convert this information into chemical signals. The Inner nuclear layer (INL), located between the ONL and the Ganglion Cell Layer (GCL), is comprised from horizontal, amacrine, bipolar, and Müller glial cells. Horizontal, amacrine and bipolar cells are interneurons that process and transmit the information from photoreceptors to the ganglion cells whereas the Müller glial cell provides a structure to the retina and maintains homeostasis. In some vertebrates, Müller glial cells can dedifferentiate into other retinal neurons as a regenerative
response to retinal injury (Lenkowski and Raymond, 2014; Ramachandran et al., 2015). The Ganglion cell layer is the innermost layer of the retina and contains retinal ganglion cells (RGCs), whose projecting axons form the optic nerve. The connection between retinal neurons mainly occurs in two different layers: the outer plexiform layer (OPL) and the inner plexiform layer (IPL) (Fig. 1.2A). All different cell types in the retina are generated from a population of multipotent retinal progenitor cells (RPCs) in a sequence that is remarkably conserved across all vertebrates studied so far (Bassett and Wallace, 2012; Cepko, 2014; Livesey and Cepko, 2001) (Fig. 1.2B). The neuronal cell differentiation in the retina occurs in an orderly manner. Ganglion cells are generated first, while rods, bipolar cells and Muller glia cells are produced at last (Cepko, 2014; Cepko et al., 1996; La Vail et al., 1991; Livesey and Cepko, 2001; Stiemke and Hollyfield, 1995; Young, 1985) (Fig1.2A). According to competence model individual RPCs are capable of committing to different cell fates depending on the influence of various extrinsic (signals contributed by the progenitor’s environment) and intrinsic (the genes expressed by that progenitor cell) factors. (Bassett and Wallace, 2012; Belliveau and Cepko, 1999; Belliveau et al., 2000; Cepko, 2014; Goetz et al., 2014; Livesey and Cepko, 2001; Marquardt and Gruss, 2002; Trimarchi et al., 2008).

1.2 Retinal cell fate determination

The very first step to produce neuronal cells of retina starts with a decision to commit a population of cells to a specific tissue fate, and this process is referred as ‘cell fate determination’. As mentioned earlier, all retinal neuronal cells derive from a multipotent progenitor cell called ‘retinal progenitor cells’. This retinal fate determination network is known to regulate not only tissue fate but also cell proliferation, pattern formation and also
Fig 1.2. Schematic representation of different cell types of retina: A. Vertebrate neural retina consists of seven neuronal cells structured in three cellular layers. ONL: outer nuclear layer, composed of rod and cone photoreceptors. INL: inner nuclear layer, composed of horizontal, Müller glial, amacrine and bipolar cells. GCL: ganglion cell layer, composed of retinal ganglion cells. The IPL: outer plexiform layer and IPL: inner plexiform layers are the regions where the connections between different retinal neurons occurs. B. The vertebrate retina differentiates in a timely manner, which is conserved across the species. Ganglion cells are the first to differentiate and Müller glial cells are the last. Modified from (Ohsawa and Kageyama, 2008).
compartment boundary establishment. Based on our current knowledge, both extrinsic and intrinsic factors are important in determining the cell fate and consecutive events during development (Fig. 1.3A). Several studies have attempted to understand the influence of various intrinsic and extrinsic factors in the cell fate decision and differentiation of retinal neurons, reviewed in (Cepko, 2014; Stenkamp, 2015). Extrinsic stimuli include secreted proteins, small molecules and cell surface factors. More specifically Sonic Hedgehog (Shh) signalling (Amato et al., 2004; Cwinn et al., 2011; Ingham and McMahon, 2001; Sakagami et al., 2009; Zhang and Yang, 2001), Fibroblast Growth Factor (FGF) signalling (Martinez-Morales et al., 2005; McCabe et al., 1999; McCabe et al., 2006; Patel and McFarlane, 2000), Notch signalling (Guruharsha et al., 2012; Jadhav et al., 2006; Riesenberg et al., 2009; Yaron et al., 2006) pathways mediates retinal differentiation by favouring or suppressing cell fate specificity/commitment of one or more population of retinal cells.

Apart from the external signalling factors, an important role is played by intrinsic factors like transcription factors that can bind to a specific DNA sequence and thereby control the expression of downstream genes (Bassett and Wallace, 2012). Among the most important ones involved in retinal cell fate determination are basic helix–loop–helix (bHLH) homeodomain and forkhead family members such as Pax6, Rax, Vsx2, Six3, Six6, Sox2, Nr2e1, Atoh7, Atoh5, Pou4f1/2/3, Isl1, Neurod1, Foxn4, Neurod4, Ptf1a, Prox1, Ascl1, Otx2, Rorb, Prdm1, Sall3, Pias3, Thrb, Rxrg, Rora, Nr2f1/2, Foxn4, Bhlhb5, Barhl2, Nr4a2, Neurod6, Satb2, Neurod2, Nrl, Nr2e3, Vsx1, Irx5, Bhlhb4, Hes1, Hes5, Hesr2. (Bassett and Wallace, 2012; Brzezinski and Reh, 2015; Brzezinski et al., 2013; Hatakeyama and Kageyama, 2004; Mao et al., 2013; Ohsawa and Kageyama, 2008; Xiang, 2013) (Fig 1.3B).
Fig 1.3. Fate determination and factors affecting during retinal differentiation: A. Retinal progenitor cells (RPCs) are multipotent, they have the competence to produce all types of retinal cells. This competence is gradually restricted and RPCs are able to produce only unidirectional sequence ensuring a unidirectional sequence of cell production, but not all progenitors at the outset of neurogenesis will produce each type of cell. In the left side, early symmetrical divisions of RPCs increase the population of progenitors. As they increase in their numbers, they produce some signals, which allow proneural gene expression and Notch-Delta mediated lateral inhibition (vertical grey arrows) to allow only a sub-population of the cells to leave cell cycle and to differentiate. Newly formed neurons/retinal cells provide signals to restrict further production of the same cell types (diagonal coloured arrow) and it also favours the generation of later neurons. As RPC attains different cell fates, the extrinsic signalling changes (vertical rectangles) and it also alters the intrinsic transcription factors expressed by RPCs (coloured nuclei). Changes in the cellular competence, intracellular signalling among progenitors and secreted signalling by differentiated/differentiating cells interact together to produce different cells. Modified from (Reese, 2011).

B. Regulation of various retinal cell fate specification by transcription factors. Combination of different transcription factors in a precise timely manner is required for the specification of retinal cell fates. Adapted from (Ohsawa and Kageyama, 2008).
Recent studies have revealed that other factors, such as noncoding RNAs and DNA-modifying enzymes, can also have substantial effects on retinal transcription and, therefore, retinal cell-fate acquisition (Busskamp et al., 2014; Carrella et al., 2015a; Chen and Cepko, 2007; Conte et al., 2010a; Conte et al., 2015; Goetz et al., 2014; Katoh et al., 2012; La Torre et al., 2013; Rapicavoli et al., 2010).

1.3 Importance of cell cycle exit in the retinal cell fate determination

Proper development of the retina relies on the precise number and timely differentiation of neuronal cells. To attain this, proliferating retinal progenitor cells should exit the cell cycle at an appropriate time. Lineage studies have shown that until the final stage of cell cycle, retinal progenitor cells remain uncommitted to a specific cell fate (Malicki, 2004). The importance of the final cell division in cell-fate determination is demonstrated by a number of findings in the past few years (Baye and Link, 2007; Farkas and Huttner, 2008; Malicki, 2004; Zhong and Chia, 2008). To gain insight into the role of final step of cell division in neuronal cell fate determination, neuronal precursors from young animals were labelled with (3H) thymidine and then transplanted into an older animal at different phases of cell cycle. It was shown that the cells transplanted at the S-phase of the cell cycle were found in the cortical layers 2/3 instead of layer 6, which would have been their neural fate. This suggests that the transplanted cells received determination signals for layer identity during the final stage of cell cycle (McConnell and Kaznowski, 1991).

There are several potential mechanisms occurring in last cell division, which could affect the retinal neuronal cell fate. The first potential mechanism during the cell cycle by which the retinal progenitors acquire different neuronal cell fate is that the expression of some retinal
cell-fate determinant factors may be activated during the final stages of cell cycle such as Ath5 and Prox1 that are turned on at the G2 phase of cycling retinal progenitors (Dyer et al., 2003; Matter-Sadzinski et al., 2005; Poggi et al., 2005). Significant evidence suggest that the stability of neural fate specification factors are tightly regulated in different phases of cell cycle (Cisneros et al., 2008). Another potential mechanism in the cell cycle which could significantly impact on the cell fate specification is the activation of cell cycle-inhibitors like CDKIs (cyclin-dependent kinase inhibitors) or down regulation of cell-cycle activators such as cyclins and CDKs (cyclin dependent kinases) (Abdullah et al., 2016; Ohnuma et al., 2002). CDKs have additional roles in cell fate determination in addition to their inhibitory activity on cell cycle (Carruthers et al., 2003; Ohnuma et al., 1999; Vernon et al., 2003). Another likely mechanism is that cell fate determining factors can down-regulate or arrest the cell cycle, thereby inducing the progenitors to commit to a specific neuronal fate. In zebrafish, Ath5 is necessary for the fate determination of RGCs. Interestingly, deletion of Ath5 skips the timing of cell cycle exit, disturbing the formation of RGCs (Kay et al., 2001). In some cases, overexpression of proneural genes like Xath5, NeuroD and Neurogenin induces cell cycle arrest and also affects cell fate specification (Farah et al., 2000; Kanekar et al., 1997; Ochocinska and Hitchcock, 2009). There are substantial evidence to suggest that forced induction of cell cycle activation by overexpression of cell cycle activators inhibits cell fate determination, whereas, cell cycle inhibition enhances cell fate determination (Calegari and Huttner, 2003; Ohnuma et al., 2002).

A number of studies have demonstrated that the dynamic of interkinetic nuclear migration and symmetry of division contribute significantly to neuronal cell fate determination. (Bilitou and Ohnuma, 2010; Cayouette et al., 2006). Time lapse imaging experiments showed that
neuroepithelial cells with greater basal nuclear migration at the final cell cycle produce post-mitotic neurons, whereas cells with more apical migrations are likely to maintain their cycling properties. These observations indicate that during interkinetic nuclear migration (INM), neuronal precursors are exposed to different extrinsic signals that may favour the fate determination or bias towards choosing a specific cell fate. The mok^309 is a zebrafish mutant caused by a nonsense mutation in the Dynactin-1 gene that regulates nuclear movement. The mok^309 mutation leads to an increase of the RGCs at the expenses of bipolar and Müller glial cells. The mok^309 mutant cell nuclei migrate more towards the basal side of the neuroepithelium and accumulate there. These cells are less exposed to Notch signalling. Since Notch activity delays retinal cell fate determination (Austin et al., 1995; Dorsky et al., 1997; Ohnuma et al., 2002), this restriction of exposure to Notch signalling increases the probability of early fate determination of initial cell types such as RGCs (Del Bene et al., 2008). The protein Syne2a, which regulates nuclear position, also has an impact on retinal cell fate determination (Tsujikawa et al., 2007).

Usually cells divide in two distinct manners at the apical side of the neuroepithelium. Some cells divide with their mitotic spindle oriented parallel to the plane of the neuroepithelium (horizontal or symmetric division), while other cells divide with their spindles oriented perpendicular to the plane of neuroepithelium (vertical or asymmetric division). In mammalian retina, vertical divisions seem to be relatively low compared to horizontal ones. However, some cells divide with the mitotic spindle oriented at random angles and not follow horizontal or vertical axes. The concept of asymmetric division in vertebrates is demonstrated by live imaging, showing that horizontal division tend to generate two daughter cells that remain
neuroepithelial in proliferative status, while vertical division produces differentiating cells (Chenn and McConnell, 1995). In the rat retina, horizontal division tend to generate daughter cells which will acquire the same cell fate, whereas a vertical division generates daughter cells with different fates (Cayouette and Raff, 2003). In the retina of some teleosts, e.g., zebrafish, vertical division is absent. However, orientation to the central-peripheral axis creates an asymmetry of the division (circumferential and radial division) (Das et al., 2003). 3D time lapse experiments carried out on zebrafish with Ath5 promoter-induced GFP expression showed that circumferential divisions tend to produce asymmetric or different fates, while radial division tend to produce symmetric or similar fates (Das et al., 2003; Poggi et al., 2005). Insuteable is a protein that helps in the proper orientation of mitotic spindles. Knockout of Insuteable (Insc) in mice inhibits horizontal divisions and activates vertical proliferative division in the retina (Siller and Doe, 2009; Zhong and Chia, 2008; Zigman et al., 2005). Also some transcription factors such as Emx2 and Pax6 are reported to regulate cellular dynamics, even if the underlying mechanisms need to be elucidated further (Estivill-Torrus et al., 2002; Gotz et al., 1998; Heins et al., 2001; Heins et al., 2002).

1.4 Role of cyclin dependent kinase inhibitors (CDKIs) and cyclins in retinal cell fate determination

Even though CDKIs have been considered as inhibitors of cell cycle, they play several additional roles, which go beyond inhibiting cell cycle. The production of postmitotic neurons and glial cells requires cell cycle arrest, cell fate determination and differentiation. As mentioned previously, these processes are controlled by both extrinsic and intrinsic factors. Some of the extrinsic factors such as Wnt, Shh, FGF and Notch are known to induce cell cycle arrest by
inducing CDKI expression (Castelo-Branco et al., 2003; Cayuso et al., 2006; Devgan et al., 2005; Li and DiCicco-Bloom, 2004; Misumi et al., 2008; Ohta et al., 2005)

During retinal cell fate determination, CDKIs activate neurogenesis and glycogenesis in a context-dependent way. In *Xenopus*, p27Xic1 is a member of the Cip/Kip CDK1 family, which co-regulates cell-cycle and retinal fate determination (Ohnuma et al., 1999). Co-overexpression of p27Xic1 with proneural genes like *Xath5* activates neurogenesis, while overexpression of only p27Xic1 activates gliogenesis in both *Xenopus* and mice (Le et al., 2006; Ohnuma et al., 2002; Ohnuma et al., 1999) suggesting that the presence of neural fate determination factors plays a key role in fate determination. To expand further, NM23-X4 (a member of NM23 family) is the binding partner of the p27Xic1 protein, which is conserved across evolution (Mochizuki et al., 2009). All members of the NM23 family are direct interactors of p27Xic1 and inhibit gliogenesis. Interestingly, mutations in the essential residues S150 and H148 of the NM23-X4 protein kinase and NDP kinase lead to inability to inhibit p27Xic1-mediated gliogenesis (Mochizuki et al., 2009).

Another role of CDKIs is on the cell fate. NM23-X4 can inhibit p27Xic1-mediated cell cycle arrest during retinogenesis (Mochizuki et al., 2009). Since NM23-X4 binding region of CDKIs overlaps with the CDK/cyclin-binding site in the N-terminus of CDKIs, the inhibition of cell cycle inhibitory action is possibly mediated by competition of NM23 binding with CDK/cyclin binding. Some studies indicated that NM3 helps to delay gliogenesis through inhibition of glial cell-fate determination and activation of cell cycle (Bilitou and Ohnuma, 2010).
Since CDKIs have been reported to act as activators of cell migration in cancer conditions (Baldassarre et al., 2005), it is also hypothesised that NM23 family members may regulate CDKI-mediated cell migration by interacting with CDKIs (Fig. 1.4).

The Retinoblastoma (Rb) protein promotes cell cycle exit by inhibiting cell division and suppresses re-entry of differentiated cells back to cell cycle (Burkhart and Sage, 2008). The phosphorylation status of Rb during the cell cycle depends mainly upon CDKs (cyclin dependent kinases), which can function by binding to cyclin proteins (Dyer and Cepko, 2001). One of the main cyclins in retinal development is Cyclin D1 (Ccnd1), which binds to Cdk4/6. Cyclin D1 is highly expressed in RPCs but after differentiation its levels go down (Barton and Levine, 2008; Dyer and Cepko, 2001; Sicinski et al., 1995). *Cyclin D1* loss in mice causes severe microphthalmia because of the reduced proliferation of RPCs (Das et al., 2009; Fantl et al., 1995). In addition, the cell cycle is prolonged and premature exit of the RPCs resulted in differentiation defects showing a higher proportion of RGCs and photoreceptors at the expense of horizontal and amacrine cells (Cunningham et al., 2002). In zebrafish, knockdown of *Ccnd1* expression results in microphthalmia with no severe defects in differentiation process (Duffy et al., 2005). Recently, association of Ccnd1 with transcriptional regulation in mouse retina development has been reported, thus suggesting multiple roles of *Ccnd1* besides cell cycle regulation (Bienvenu et al., 2010).

The role of other cyclins, including Ccnd2 and Ccnd3 and Cyclin E play not so crucial roles in the retina, however, there are reports marking the role of Cyclin E in rescuing the RPC defects during ablation of *Ccnd1* (Carthon et al., 2005; Das et al., 2009; Geng et al., 1999).
After analysing the currently known data, it looks like the balance between the levels of cell cycle activators (cyclin-CDKs) and repressors (CDKI) is required to direct the cell cycle exit and retinal cell fate specification in a proper way (Miles and Tropepe, 2016).

Fig 1.4. Roles of CDKIs in regulating cell cycle, cell fate determination, differentiation and migration: CDKIs are induced by multiple differentiation factors. CDKIs regulate three major events by interacting with multiple factors. The N-terminal of the CDKIs bind can inhibit CDK/cyclin complex, N-terminus also regulates cell fate determination. The C-terminus regulates differentiation and migration by interacting with cytoskeletal factors. NM23 (a binding partner of CDKI) by binding to CDKI is likely to influence all three major mechanisms mediated by CDKI. Adapted from (Bilitou and Ohnuma, 2010).
1.5 Photoreceptor and their cell fate specification in vertebrates

The visual process starts in photoreceptors which are the only neurons able to capture light stimuli. The chemical message of photoreceptors is processed by interneurons (bipolar, horizontal and amacrine cells) and conveyed to visual centres in the brain by ganglion cells. Vertebrate photoreceptors includes an outer segment filled with photoreceptive pigments (opsins), a connecting cilium, inner segment, the nucleus, the axon and the synaptic terminal. Normal photoreceptor function depends upon maintaining these subcellular compartments (Brzezinski and Reh, 2015; Swaroop et al., 2010) (Fig.1.5). The energy demanding nature of photoreceptors in visual function makes these cells irreplaceable and more prone to degenerative disorders. There are two subtypes of photoreceptors, i.e., rods and cones. Cone photoreceptors are sensitive to bright light and contain different opsins which mediate the colour vision. Conversely, rod photoreceptors are highly sensitive and function in low light conditions and are mainly responsible for night vision.

Photoreceptors are generated by a multipotent proliferative progenitors. There is a large body of experimental evidence that suggests a correlation between the timing of cell cycle exit of the progenitors and cell fate determination (Wong and Rapaport, 2009; Young, 1985). Cone photoreceptors are formed before the initiation of rod photoreceptor production (Fig. 1.2B) (Carter-Dawson and LaVail, 1979). Large number of cell lineage tracing studies demonstrate that progenitors are not limited to produce single type of retinal neuron (Turner and Cepko, 1987; Turner et al., 1990; Wets and Fraser, 1988) or in other words there are no progenitors which can produce exclusively photoreceptors. However, it has been shown that some teleost
(zebrafish and goldfish) possess a progenitor, which can exclusively produce rod photoreceptors, even though it is considered as an adaptation to maintain light sensitivity (Raymond and Rivlin, 1987). Some studies have shown the presence of cone photoreceptor-specific progenitors in fish (He et al., 2012; Suzuki et al., 2013). Six6 overexpression induced by NeuroD stimulates retinal progenitor cells (RPCs) proliferation and impairs photoreceptor differentiation with no noticeable changes in other retinal cells in medaka (Conte et al., 2010b).
Analysis of the currently available evidence suggests that most of the molecular events involved in the generation of photoreceptors and their subtype can either occur in proliferative progenitors or postmitotic precursors.

Otx2 (orthodenticle homeobox 2) is a key transcription factor necessary for photoreceptor development. It belongs to the paired homeobox family. The early expression of Otx2 in the progenitors helps the cells to acquire photoreceptor and bipolar cell identity. Deletion of Otx2 results in inhibition of development of these two cells whereas overexpression results in overproduction of these two cell types (Koike et al., 2007; Nishida et al., 2003; Sato et al., 2007; Wang et al., 2014). Importantly, other transcription factors, such as Vsx2 (Chx10), Prdm1 (Blimp1) are activated by Otx2 and are necessary for the fate determination of photoreceptors. These two factors control the fate of Otx2-expressing cells. Vsx2 acts negatively on the photoreceptor development by suppressing photoreceptor-specific genes (Dorval et al., 2005; Livne-Bar et al., 2006). Otx2 has been reported to activate Prdm1 in a direct manner (Brzezinski et al., 2010; Wang et al., 2014). Prdm1 prevents Otx2 expressing cells from attaining bipolar cell fate. In Prdm1 mutants, Otx2 expressing cells begin to develop photoreceptor identity but later switch to a bipolar cell fate (Brzezinski et al., 2010; Brzezinski et al., 2013; Katoh et al., 2010) (Fig. 1.6A).

Recent research, carried out by lineage tracing studies, has revealed a subset of proliferative progenitors that show restricted cell fate and express different combination of transcription factors. The fate mapping of a subset of progenitors expressing the transcription factor Ascl1 can potentially become all the retinal cell types except RGCs suggesting that expression of Ascl1 can restrict the fate of single cell type in differentiating neural retina (Brzezinski et al., 2011).
Additionally, another study conducted on murine RPC discloses the varying expression of the gene \textit{Olig2} across time. When the daughter cells of E13.5 to E14.5 \textit{Olig2} expressing RPCs were clonally labelled by retroviral infection, only cones and horizontal cells were marked. When day P0 or P3 \textit{Olig2} expressing RPCs were marked by viral infection, only rods and amacrine cells were labelled. The RPC clones lacking \textit{Olig2} were comprised of rods and bipolar cells, as well as rods and Muller glial cells (Wang and Cepko, 2016). According to other reports, the progenitors expressing transcription factor, Olig2, can give rise to all cells except RGCs and Müller glia (Hafler et al., 2012; Jeon et al., 1998) (Fig 1.6B).

\textit{Pax6} is a very essential gene expressed in all the retinal progenitors. The absence of \textit{Pax6} in the retina leads to a failure in the formation of photoreceptors as well as of other cell types (Marquardt et al., 2001). Notably, \textit{Crx} and other photoreceptor specific genes were dramatically upregulated in \textit{Pax6} mutants (Oron-Karni et al., 2008). These data suggest that \textit{Pax6} expression is essential for the progenitors to commit towards photoreceptor (and other kind of cells) fate but more interestingly, it also restricts the capability of progenitors to trigger photoreceptor program. Similarly, functional studies on other transcription factors such as Ascl1 (Mash1), Foxn4, Rorβ, Atoh7 (Math5), Neurod1, Thrb2 (Thrb) indicate the prominence of these factors in molecular mechanisms of photoreceptor development/maintenance. Reviewed in (Brzezinski and Reh, 2015). \textit{Crx} (cone rod homeobox) gene acts downstream of \textit{Otx2} and is required for the accurate expression of almost all photoreceptor genes (Chen et al., 1997; Freund et al., 1997; Furukawa et al., 1997). Depletion of \textit{Crx} leads to severe reduction in expression levels of photoreceptor specific genes (Samuel et al., 2014). Since \textit{Crx} is expressed both in rod and cone
Fig 1.6. Regulation of photoreceptor fate determination: A. The cells expressing Otx2 can acquire the potential to become photoreceptors and bipolar cells. Otx2 induced activation of Prdm1 and Vsx2 expression restrict bipolar and photoreceptor potential respectively. B. A group of progenitor cells expressing Vsx2 and Pax6 have the potential to generate all kinds of retinal neurons. A subset of these progenitors loses the capacity to become RGCs (retinal ganglion cells) by expressing Ascl1. Among these progenitors, expression of Olig2 and/or Otx2 can give rise to all the retinal cells except RGCs and Müller glia cells. Adapted from (Brzezinski and Reh, 2015).
photoreceptors, its deletion/mutation can result in diseases that can affect both rods and cones or either of them, for example Leber's Congenital Amaurosis (LCA) and cone-rod dystrophy (Freund et al., 1997; Swaroop et al., 1999; Tran and Chen, 2014).

The decision between rod and cone fate mainly depends upon the action of either positive regulators those insist a fate choice (cone or rod) or because of negative factors that inhibit the fate choice of a particular cell type thereby facilitating the fate determination of another cell type. In majority of the cases the combination of positive and negative regulators results in the ‘fate determination’ of rods and cones.

Some studies conducted on mice suggest that a bipotential photoreceptor precursor is established soon after cell cycle exit (Fig. 1.7A) (Swaroop et al., 2010). According to this study, precursors can be affected by Nrl and can take on rod fate. There is evidence to support this model such as the overlap between the expression of some rod and cone specific markers during development (Nrl and Thrβ2) (Ng et al., 2011). Expression of Nrl in cones can induce cone cells to move towards rod fate partially (Oh et al., 2007). Removing Nrl from adult rods can partially convert them towards a cone identity (Montana et al., 2013).

According to another theory another factor upstream to Nrl is responsible for the commitment of progenitors to rod photoreceptors, after the cell cycle exit (Emerson et al., 2013) (Fig. 1.7B). According to this model, Nrl expression in precursors would repress cone development and affect rod maturation. This model can be strengthened by the fact that in Nrl mutants photoreceptor specification is unaffected (Daniele et al., 2005).
The two key factors regulating the choice of rod over cone or vice versa are located and acts downstream of Otx2 and Crx, and the two key factors controlling this event are Rorβ and Nrl (Swaroop et al., 2010). Otx2 together with Rorβ activates the expression of the transcription factor Nrl in a subset of newly-formed postmitotic precursors, which will later develop into rod photoreceptors (Akimoto et al., 2006; Fu et al., 2014; Kautzmann et al., 2011; Roger et al., 2014). Depletion of either Rorβ or Nrl results in the absence of rod photoreceptors and the formation of S-opsin expressing cones (Fu et al., 2014; Mears et al., 2001). Furthermore, Nrl also activates several rod specific genes including Nr2e3, a transcription factor that can activate rod genesis and suppress cone-specific genes (Chen et al., 2005; Cheng et al., 2006; Cheng et al., 2011; Cheng et al., 2004; Hao et al., 2012). Nr2e3 mutants display significant rod defects along with reduced rod-specific gene expression and excess of S-opsin expression (Akhmedov et al., 2000; Corbo and Cepko, 2005). Overall these data indicates that attaining normal development of rod photoreceptor requires the combination of both the rod activator Nrl and the repressor Nr2e3.

Even though there are many studies conducted to understand the transcription factors network in rod fate determination, much less is known about cone specification. However, some cone-specific markers are characterized at retinal development during embryonic stages. There are three possible explanations by which cone photoreceptor fate specification can be explained. First, and as mentioned above, commitment of a progenitor towards cone fate might be a default outcome, requiring only negative regulators such as Nr2e3 or Notch signalling to produce non-cone cell fates (Fig. 1.7A,C). The second possibility is that a cone-specific factor can activate a set of progenitors to commit towards cone fate and/or suppress rod fate like Nrl.
(Fig. 1.7D). However, the precise identification of such cone-specific factors remains mostly unclear. The third possibility is the combined action of more widely expressed regulators, which are not only restricted to produce cones but also other cell fates (Fig. 1.7E).

The thyroid hormone receptor Thrβ2 is one of the transcription factors involved in cone photoreceptor specification. In mice, blue cone and green cone formation depends upon the predominant expression of S-opsin or M-opsin. Thrβ2 deficient mice lack M-subtypes (Ng et al., 2001; Roberts et al., 2006). However, Thrβ2 null mice still produce cones, indicating that Thrβ2 is not necessary for the commitment to cone identity. Another transcription factor Rxry (Rxrg) is also expressed in developing cones and ganglion cells (Mori et al., 2001). Loss of Rxry showed no effect on the initial phase of cone formation (Roberts et al., 2005). In this mutant, M-opsin expressed normally and S-opsin was expressed robustly by all cones. Therefore, during cone and its subset formation Thrβ2 acts as a positive factor to express M-opsin, and it also acts as a negative regulator along with Rxry to suppress S-opsin. The forced expression of Onecut1 results in the formation of excess cones at the expense of rods (Emerson et al., 2013). Furthermore, it was also shown that Onecut1 and Otx2 collectively activate Thrβ2 expression, suggesting the combined effect of these two transcription factors is essential to acquire a cone fate. Mice lacking Neurod1 fail to express Thrβ2 and showed cone subtype defects (Liu et al., 2008). Soluble molecules such as taurine and retinoic acid were found to promote Rhodopsin expression in cultured retinal cells (Altshuler et al., 1993; Kelley et al., 1994).

Other factors like Shh and activins also promote Rhodopsin expression in vitro (Davis et al., 2000; Levine et al., 2000; Levine et al., 1997). Besides these soluble factors, also the Notch signalling pathway plays
Fig. 1.7. Models of photoreceptor fate specification. **A.** A bipotent Otx2 expressing precursor will become a cone (c) by default except the expression of Nrl commits the precursor towards rod (R) fate. **B.** Instead, precursor can commit to rod fate because of the factor present upstream of Nrl. In this case, Nrl would favour the rod maturation whereas inhibiting the expression of cone-specific genes. **C.** Blue (Opn1sw-expressing) cone (Cb) fate might be the default outcome of Otx2-expressing cells. According to this theory, additional factors (X, Y, Z) are needed to suppress blue cone fate and to promote other fates including green cones (Opn1mw-expressing) (Cg). **D.** This model proposes that cone development is not a default consequence, instead, may require the action of some discrete factors expressed only by the cones. **E.** Commitment of the precursors towards cone fate might be the outcome of intersection of some common factors for example: Otx2 expressing precursors exposed to the same signal (Z) can acquire different fates depending on the co-expression of other factors like X or Y. Adapted from (Brzezinski and Reh, 2015).
a crucial role in transition of multipotent progenitors to photoreceptor precursor (Cepko, 2014; Nelson et al., 2007; Perron and Harris, 2000; Xiang, 2013). Otx2 expression is one of the earliest sign of photoreceptor transition. Inhibition of Notch signalling causes the expression of Otx2 in most progenitors in mouse retina (Jadhav et al., 2006; Nelson et al., 2006; Yaron et al., 2006) whereas conditional deletion of components of the Notch pathway reduces the number of progenitors and increases the number of rod and cone photoreceptor precursors (Jadhav et al., 2006; Mizeracka et al., 2013; Riesenberg et al., 2009; Yaron et al., 2006). Even though the above evidence strongly corroborates the inhibitory role of the Notch pathway in photoreceptor genesis, yet the exact mechanisms are obscure until so far and further investigation is needed in this regard.

1.6 MicroRNAs (miRNAs)

MicroRNAs (miRNAs) are recognised as fundamental players in eukaryotic gene expression (Bartel, 2009). They are endogenous 20–24 nucleotide long non-coding RNAs that can bind to target mRNAs of protein-coding genes and thereby direct posttranscriptional silencing either through transcript degradation or translational repression (Ebert and Sharp, 2012; Pasquinelli, 2012). They were discovered over 2 decades ago in C. elegans (Lee et al., 1993). They have found to be present in plants and animals (Lagos-Quintana et al., 2001; Lee and Ambros, 2001; Pasquinelli et al., 2000). MiRNAs bind to sequence motif predominantly localized in the 3’ untranslated regions (UTRs) of their target mRNAs, even though there are some examples of target sites in coding regions (Tay et al., 2008). A single miRNA can recognise several hundreds of target genes and, on the other hand, a gene can have target sites for different miRNAs (Peter, 2010; Wu et al., 2010; Yoo et al., 2009).
MiRNA transcriptional units can be intergenic or intragenic in terms of location with respect to other genes. Intragenic miRNAs usually reside in the introns of either coding or non-coding transcripts (host genes) but in rare instances they can localize to exonic regions. Intragenic miRNAs tend to share the promoter and other regulatory sequences of their host genes. Quite often several miRNAs reside in close vicinity to each other, creating a polycistronic transcription units (Lee et al., 2002). The miRNAs present in the same cluster are usually co-transcribed. However, miRNA genes often have multiple transcription start sites (Ozsolak et al., 2008). MiRNA genes are transcribed by RNA polymerase II enzyme and controlled by RNA polymerase II associated transcription factors and epigenetic regulators (Cai et al., 2004; Lee et al., 2004). After transcription, miRNA genes give rise to long primary transcripts with a hairpin structure that is known as primary miRNA (pri-miRNA). Usually pri-miRNA consists of a stem, a terminal loop and single stranded RNA segments at both 5’ and 3’ ends (Ha and Kim, 2014). The RNase Drosha, along with its cofactor DGCR8, forms a protein complex called microprocessor. In the canonical miRNA biogenesis, pri-miRNA structures are recognised by the microprocessor complex (Denli et al., 2004; Gregory et al., 2004). The two double stranded RNA binding domain (dsRBD) of DGCR8 recognise pri-miRNA and its C terminus interacts with Drosha (Han et al., 2006; Yeom et al., 2006). Drosha cleaves the hairpin nearly about 11 bp away from the basal junction and approximately 22 bp away from the apical junction linked to the terminal loop and generates a two nucleotide overhang at 3’ end (Han et al., 2006; Zeng et al., 2005). Finally this mechanism leads to the formation of a small hairpin shaped RNA molecule of around 70-100 nucleotide length called precursor miRNA (pre-miRNA) (Lee et al., 2003). Following Drosha processing, pre-miRNAs are exported to the cytoplasm by a protein called Exportin 5 by
forming a transporter complex with GTP binding nuclear protein RAN.GTP and a pre-miRNA
(Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003). Upon export to the cytoplasm, the pre-
miRNA is recognised and cleaved by Dicer near the terminal loop liberating a small RNA duplex
(Bernstein et al., 2001; Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001; Knight
and Bass, 2001). One of the strands of RNA duplex (mature miRNA) interacts with AGO protein
to form an effector complex called miRNA induced silencing complex (miRISC), whereas the
other strand is degraded (Mourelatos et al., 2002; Tabara et al., 1999). Several studies suggest
that the strand with less stable base pairing with the 5’ end is chosen as guide strand (Khvorova
et al., 2003; Schwarz et al., 2003). The main components of miRISC are AGO protein family
members that directly bind to the miRNA and GW182 family proteins which mediates
translational repression or/and mRNA decay. This occurs in the processing bodies (P-bodies),
which are the cytoplasmic foci that are induced by mRNA silencing and decay but are not
necessarily required for miRNA-induced gene silencing (reviewed in (Lin and Gregory, 2015). In
the mature miRNA, the bases located in position from 2 to 7 are referred as ‘seed’ and these
are believed to play the most important role in recognising the complementary binding sites of
target mRNAs (Bartel, 2009). In most cases, binding of the miRNA ‘seed’ is perfectly
complementary to the target region of mRNA and it leads to either degradation of target mRNA
or translational repression (Bartel, 2009). The degradation process includes the involvement of
deadenylase complexes such as CCR4-NOT complex that shorten poly (A) tails. Shortening of
poly (A) tails induces a process called de-capping, i.e., removal of 5’ cap. After this process
mRNAs are removed from the cell by the action of 5’ to 3’ exoribonucleases such as Xrn1
(Huntzinger and Izaurralde, 2011). Studies on miRNAs conducted over the past 2 decades
Fig. 1.8. Mechanism of miRNA biogenesis: In the nucleus, MicroRNA (miRNA) genes are transcribed as primary miRNAs (pri-miRNAs) by RNA polymerase II (Pol II). Primary-miRNAs are cleaved by Microprocessor, which includes Drosha and DGCR8, to produce the 60–70-nucleotide precursor miRNAs (pre-miRNAs). The pre-miRNAs are then exported from the nucleus to the cytoplasm by exportin 5 (XPO5) and further processed by Dicer, a ribonuclease III (RIII) enzyme that produces the mature miRNAs. One strand of the mature miRNA (the guide strand) is loaded into the miRNA-induced silencing complex (miRISC), which contains Dicer and Argonaute (AGO) proteins, directs the miRISC to target mRNAs by sequence complementary binding and mediates gene suppression by targeted mRNA degradation and translational repression in processing bodies (P-bodies). TRBP, transactivation-responsive RNA-binding protein. Adapted from (Lin and Gregory, 2015).
suggest that miRNAs are present in almost all the tissues and play a role in various cellular mechanisms. MiRNAs are estimated to regulate the translation of more than 60% of protein-coding genes (Esteller, 2011). They are involved in the regulation of numerous biological processes including proliferation, differentiation, and apoptosis (Bartel, 2004; Rodriguez et al., 2004). Disrupting the global miRNA biogenesis by ablating Dicer in mice results in embryo death before gastrulation (Bernstein et al., 2003). Earlier studies based on Dicer deletion, one of the core components of miRNA biogenesis demonstrated the essential roles of miRNAs in many neural cell types during different stage of development. Reviewed in (Sun et al., 2013). In mice disruption of one of the Ago family members Ago2 results in embryonic lethality early in development after the implantation stage (Liu et al., 2004; Morita et al., 2007). Conditional depletion of DGCR8 in mouse kidney leads to severe hydronephrosis, kidney cysts, progressive renal failure and premature death within the first two months after birth (Bartram et al., 2015). In mouse brain, miR-124 is expressed specifically and abundantly (Hemming et al., 1989). In *Xenopus laevis* miR-124a contributes to the intrinsic regulation of RGC growth cone aging and thereby ensures proper axonal targeting to the tectum (Baudet et al., 2012). Furthermore, knockdown of miR-124a leads to an increase in apoptosis and up-regulation of the Caspase9 protein in the eyes of *Xenopus laevis* (Walker and Harland, 2009) underlying the importance of miR-124a in regulation of programmed cell death. A variety of miRNAs are reported to regulate circadian rhythms, including: miR-26a, miR-182, miR-124, miR-107, miR-155, miR-16, miR-181a, let-7a (Chen et al., 2014; Curtis et al., 2015; Daimiel-Ruiz et al., 2015; Figueredo Dde et al., 2015; Han et al., 2016; Shi et al., 2009; Zhang et al., 2016). MiR-1 is one of the most highly expressed miRNAs in skeletal and heart muscle across a wide range of
species from *Drosophila melanogaster* to humans (Kwon et al., 2005; Lagos-Quintana et al., 2002; Sempere et al., 2004). In the mice miR-1 regulates the cardiac gene program during the transition from prenatal to neonatal stage (Wei et al., 2014).

### 1.7 MicroRNAs (miRNAs) and disease

Because of the enormous influence of miRNAs on biological processes, their dysregulation may play a pathogenic role in human diseases. There is a large bulk of evidence revealing the involvement of miRNAs in human pathology. More recently, miRNAs are also used as biomarkers in the disease manifestations (Weiland et al., 2012). The involvement of miRNAs in human pathology could be either direct or indirect. Mutations affecting the miRNAs functions include: mutations in the miRNA itself (either point mutation in the mature sequence or deletions/duplications of the entire miRNA locus), mutations in the 3’ UTR of target mRNAs that can result in disruption or in the generation of aberrant target sites for a given miRNA, mutations in the genes which take part in miRNA biogenesis and function, hence, affecting the global function of miRNAs (Meola et al., 2009). MiRNA dysregulation is described in many neurological disorders. MiR-19, miR-101 and miR-130 levels, modify the penetrance of spinocerebellar ataxia type 1 by the co-regulation of ataxin 1 levels (Lee et al., 2008). Mutations in the seed region of miR-184 cause severe keratoconus combined with early-onset anterior polar cataract (Hughes et al., 2011). Two different point mutations in the 3’UTR of REEP1 alters the sequence of predicted target site for miR-140, which is associated with an autosomal dominant form of hereditary spastic paraplegia (SPG31) (Beetz et al., 2008; Zuchner et al., 2006). DiGeorge syndrome patients are characterized by the deletion of the DGCR8 gene (Shiohama et al., 2003). Overexpression of miR-195 can provoke heart dilative hypertrophy in
mice. Interestingly, miR-195 levels are raised in failing human hearts (van Rooij et al., 2006). Decreased levels of either miR-133 or miR-1 can initiate cardiac hypertrophy (Care et al., 2007; Sayed et al., 2007). More recently, miR-221 is reported to be dysregulated in acute coronary syndrome (Coskunpinar et al., 2016). Renal miR-196a/b levels go down rapidly during unilateral ureteral obstruction (UUO)-induced renal fibrosis in mouse (Meng et al., 2016). Depletion of DGCR8 in mice causes infertility due to multiple abnormalities in uterus (Kim et al., 2016) hereby underlying the importance of miRNAs in normal development and function of reproductive organs. The first detailed miRNAs profiling in multiple cancer samples showed that the changes in miRNAs correlate with the developmental lineage and differentiation states of many cancers (Lu et al., 2005).

MiRNAs are also involved in cancer, with a role both in primary tumorigenesis as well as in formation of metastasis. The miR-17-92 cluster contains the first identified oncogenic miRNAs in mammals (oncomiR) (He et al., 2005). MiR-10b is the first reported metastasis promoting miRNA (Ma et al., 2007). Furthermore miR-15a and miR-16-1 were shown to inhibit tumorigenesis by targeting the Bcl2 oncogene (Kent and Mendell, 2006). Recently roles of miR-193a-3p and miR-193a-5p were showed in osteosarcoma, as these miRNAs suppressed osteosarcoma cell migration and invasion (Pu et al., 2016).

1.8 MiRNAs in eye development and function

As mentioned before, miRNAs regulates many important biological processes, which include cell growth, differentiation and development and death. There is strong evidence that miRNAs are part of the gene regulatory network that ensures proper physiological development and function of the eye. Any minor defect in any of the members of this network may result in
disorders affecting development and function of the eye. Recent studies on the miRNA-controlled gene networks revealed that miRNAs may represent molecular biomarkers and therapeutic agents in eye disorders.

Early studies on the analysis of miRNA expression in the eye started by a cloning and Sanger sequencing-based strategy that identified small RNAs expressed in the newt eye whereas miRNA arrays were used to identify the expression of miRNAs in mouse cornea, lens and different retinal layers (Makarev et al., 2006; Ryan et al., 2006). Detailed microarray analysis identified 78 retinal miRNAs with enriched expression in the murine retina compared to other tissues and cell types (Xu et al., 2007). Furthermore, thorough expression investigation of eye enriched miRNAs provided the first deep knowledge of their spatiotemporal distribution by RNA in situ Hybridization in the murine eye (Karali et al., 2007). Later on, a detailed survey of miRNA expression in ocular tissues by combined procedure of microarray analysis and RNA in situ hybridization (ISH) revealed detailed expression profiles of 122 miRNAs, with the majority of them expressed in one or more cell layers of the neural retina (Karali et al., 2010) and this expression atlas is publicly available (http://mirn-eye.tigem.it). Another miRNA array approach is used in order to monitor the miRNAs profile during retinal development (Hackler et al., 2010). Recently, quantitative analysis of the retinal miRNA transcriptome by next-generation sequencing (NGS) identified miRNAs showing light-dependent expression pattern (Krol et al., 2010). Until recently, information on the miRNA transcriptome (miRNome) in the human retina was not available. Then my lab carried out the first study on the human retina miRNome starting to unveil its complexity. Notably, analysis of the expression levels of miRNAs and their variants in 16 healthy neural retinas by small RNA-seq leads to the identification of 51 novel
putative miRNAs (Karali et al., 2016) and the data generated through this study is publicly available at (http://miretina.tigem.it).

Conditional Dicer knockout mouse showed impairment in the normal development of retina, lens, cornea and optic chiasm because of the global perturbation of miRNA function (Damiani et al., 2008; Davis et al., 2011; Georgi and Reh, 2010; Pinter and Hindges, 2010). In cataract patients, 110 abundant miRNAs are identified from aqueous humour. Among them, miR-202, miR-193b, miR-135a, miR-365 and miR-376a are the most abundant miRNAs (Dunmire et al., 2013). The downregulation of let-7c, miR-29a and miR-29c are found to be involved in progression of cataract in Shumiya cataract rats whereas, these miRNAs are upregulated in non-cataractous lenses (Kubo et al., 2013). In addition to this, in lens epithelial cells (LECs) of a human donor capsular bag model epithelial-to-mesenchymal transition (EMT) is regulated by miR-204 by targeting SMAD4 (Wang et al., 2013). More valid evidence of direct involvement of miR-204 in eye pathogenesis emerged in the year of 2015 from our lab with the identification of a point mutation in the ‘seed’ region of miR-204. The disease is characterised by ocular coloboma associated with retinal degeneration, also with the presence of cataract in some of the affected individuals. In vivo studies on medaka fish demonstrated that the contribution of this mutation to the observed eye pathology is more likely relies on a gain-of-function mechanism (Conte et al., 2015). This represents the first example of a retinal dystrophy caused by mutation in a miRNA and also uncovers a newly identified role of miR-204 in photoreceptor survival and function. Hence miR-204 constitutes a novel target for therapeutic intervention in eye disorders.
1.9 The miR-204/211 family

It is important to mention that miR-204 has a very close paralog, miR-211, in mammals. They are located in the intronic region of the \textit{TRPM3} and \textit{TRPM1} genes respectively. MiR-204 and miR-211 share the same seed sequence and their mature sequence differs by one or two nucleotides depending on the species. Since they share common seed region, most likely they may regulate the same set of target genes (Lewis et al., 2005). In lower vertebrates such as medaka fish, miR-204 is present in two identical copies (Conte et al., 2010a). In mammals, one of the copies of miR-204 is evolved to become miR-211.

MiR-204 exhibits a distinct and evolutionary conserved predominant expression in the eye, especially in the RPE, neural retina, lens and ciliary body in fish, mouse and human (Conte et al., 2010a; Conte et al., 2015; Deo et al., 2006; Karali et al., 2010; Karali et al., 2007; Shaham et al., 2013; Xu et al., 2007). MiR-204 is extensively investigated in our lab. During the last decade our lab has contributed the major body of knowledge concerning the functional contributions of miR-204 in eye development and pathogenesis. The first ever characterization of miR-204 function by using an in vivo model was performed in our lab. Knockdown of miR-204 in medaka fish resulted in a severe eye phenotype characterised by microphthalmia, abnormal lens formation and altered dorsoventral (D-V) patterning of the retina, which is associated with optic fissure coloboma. We showed that miR-204, by modulating the expression of the target gene \textit{Meis2}, regulates lens and retinal development in medaka fish. More interestingly, we also found that the pathway which involves the transcription factor Pax6, a “master regulator” of eye development is perturbed and we also showed that miR-204 can indirectly repress Pax6 by targeting \textit{Meis2} (Conte et al., 2010a). Remarkably, further research on miR-204 showed
that Pax6 controls the expression of miR-204. This study identified new set of miR-204 targets like Sox11 (Shaham et al., 2013). Our further investigation is a step towards understanding the networks operated by miR-204 in cell migration, which is a key process in embryo morphogenesis. In particular, we found inability of migration in neural cell crests (NCCs) upon ablation of miR-204 in medaka. By additional experiments, we found that Ankrd13A, which is a direct functional target of miR-204 modulates the lens cell migration by interfering with cytoskeleton and focal adhesion formation (Avellino et al., 2013). Our lab did a transcriptome analysis by manipulating the expression of miR-204 in order to gain insight in to the possible role of miR-204 in vertebrate organogenesis. With the combined approach of RNA-seq and differential expression analysis we analysed putative targets of miR-204, after its overexpression and knockdown and we identified a broader range of miR-204 target genes belonging to different biological pathways. Then, we demonstrated in vivo (medaka fish) that altered expression of miR-204 is linked with abnormal axonal projection of retinal ganglion cells to the brain or in axon guidance by directly targeting EphB2 and Efnb3 (Conte et al., 2014). MiR-204 and miR-211 are the most highly expressed miRNAs in the RPE (Wang et al., 2010). Comparison of miRNA expression profiles of differentiated primary human fetal RPE (hfRPE) cells to dedifferentiated hfRPE cells showed an opposite trend in the expression levels of miR-204/211. MiR-204/211 expression levels are upregulated during differentiation while they are downregulated during dedifferentiation of hfRPE cells. The Microphthalmia-associated transcription factor (MITF), a key regulator of RPE differentiation, is the target gene of miR-204/211 involved in this process (Adijanto et al., 2012). The Forkhead box C1 (FOXC1) is a transcription factor that controls eye development (Kidson et al., 1999). In human tabular
meshwork (TM) cells, miR-204/211 causes the downregulation of FOXC1 and of its targets, among which *Meis2* is the important one. Interestingly *Meis2* is also a target of miR-204 in lens development and dorsoventral (D-V) patterning of retina (Conte et al., 2010a). This data suggested a pathway that involves an interaction between FOXC1, miR-204 and *Meis2*. As discussed previously, miR-204 levels are upregulated in a mouse model of posterior capsule opacification (PCO). Detailed studies identified *Meis2* as a target gene involved in the observed phenotype and the involvement of miR-204 in epithelial to mesenchymal transition (EMT) in lens epithelial cells (LECs) (Hoffmann et al., 2012; Wang et al., 2013).

1.10 MiRNAs in photoreceptors

Emerging evidence suggests that miRNAs are fundamental players in cellular stress. Conditional knockout (cKO) of Dicer1 in mature rod photoreceptors leads to progressive, early-onset retinal degeneration and functional impairment of photoreceptors in mice. The early morphological phenotype includes disorganization of outer segments starting at 8 weeks of age. The symptoms progress and there is a complete loss of photoreceptor nuclei by 14 weeks of age. Before degeneration, these cKO showed no abnormalities in phototransduction and visual cycle, suggesting that the loss of rod photoreceptor miRNAs caused a primary defect in rod photoreceptors and this resulted in the defect of other neurons (Sundermeier et al., 2014). Targeted disruption in the activity of miR-124a in mice showed abnormalities in the CNS (central nervous system), including cone photoreceptor death. The striking phenotype in the photoreceptor includes the reduction in the number of cone photoreceptors by apoptosis and mis-localization of TRβ2 (an early cone marker) positive cells, but the proliferation is unaltered.
More interestingly, quantitative measurements of some of the early neurogenesis genes such as *Neurod1, TRβ2, Crx, Otx2* and *Ngn2* were unaltered. Findings of this study suggest that miR-124a is essential for the survival and proper localization of cone cells and its role is most likely exerted after the neurogenesis and not during early neurogenesis (Sanuki et al., 2011).

One of the most abundant miRNAs in the mature retina is the miR-183 cluster comprised of miR-96, miR-182 and mir-183. In the retina these miRNAs are enriched in both cone and rod photoreceptors (Karali et al., 2007; Xu et al., 2007). They are light regulated, as the levels of miR-183/96/182 are upregulated in light adapted and are downregulated in dark adapted retinas (Krol et al., 2010). The expression levels of these miRNAs are downregulated in P347S retinitis pigmentosa mouse model (RP) (Loscher et al., 2008). A sponge transgenic mouse model characterized by the silencing of miR-183/96/182 activity resulted in a bright light induced retinal degeneration by targeting one of the apoptotic factor Caspase2, underlying the protective role of these miRNAs against light stress (Zhu et al., 2011). The importance of the miR-183/96/182 cluster in photoreceptors is further confirmed by the results of the disruption of this cluster by using a gene-trap embryonic stem cell clone. The inactivation of these miRNAs resulted in an early-onset and progressive photoreceptor degeneration leading to alteration in visual functions. In addition, knockout mice showed an alteration in the expression profiles of the genes involved in synaptogenesis, synaptic transmission and phototransduction, indicating the role of miR-183/96/182 in synaptic connectivity of photoreceptors (Lumayag et al., 2013). In mice, global inactivation of miRNA processing in cone photoreceptors, after the complete development of the retina, leads to the gradual loss of the cone outer segments and cone dysfunction. However, the number of cones and cell
bodies remain unchanged. Most importantly, the re-expression of the sensory-cell specific miR-182 and miR-183 in cones prevented outer segment loss (Busskamp et al., 2014). Several pieces of evidence, strongly suggests that miR-183/96/182 family is mostly involved in the maintenance of photoreceptor outer segments (Krol et al., 2015).

In Drosophila photoreceptors, miR-7 regulates the cell fate decisions by acting in a reciprocal negative feedback mechanism with a transcription factor. Expression of miR-7 is activated in the differentiating photoreceptor cells. It regulates the transcription factor Yan through EGF receptor mediated signalling mechanism. The discovery of this study concluded that miR-7 neither plays a direct role in cone photoreceptor development nor did it promotes the cone photoreceptor fate. Perhaps, it promotes overall photoreceptor fate at the expense of a cone fate (Li and Carthew, 2005).

One of the early studies addressing the role of miRNAs in photoreceptors is the conditional knockout of Dicer in the retina in mice. In these animals, by 1 month of age the response to light was significantly reduced. The retina of these mice showed no visible phenotype on the retinal morphology and function during early postnatal stages. The striking phenotype includes inability to respond to light along with morphological defects such as the formation of photoreceptor rosettes. Further on, these mice showed concomitant decrease in both scotopic and photopic ERG responses. Removal of only one allele of Dicer led to no morphological abnormalities. However, functional aspects such as ERG remained low throughout life (Damiani et al., 2008).

The circadian regulation of the photoreceptors mainly includes shedding and renewal of outer segment discs, neurotransmitter release and maintenance of the ion channel activity (Ivanova
and Iuvone, 2003; Ko et al., 2001; Ko et al., 2007; Reme et al., 1986). As mentioned in the other part of this section, miRNAs are also involved in the regulation of circadian rhythms. One such example is miR-26a. A study conducted on chicken has showed that miR-26a is a key regulator of L-type voltage gated calcium channel α1C subunit (L-VGCCα1C) expression in cone photoreceptors. In photoreceptors L-VGCCα1C is activated under circadian control, with higher levels at night and lower during the day (Shi et al., 2009).

Photoreceptor cells are physically in contact with RPE and depend heavily on them for their proper function. Therefore, perturbations of the RPE can lead to the dysfunction of photoreceptors. Among the most striking examples are age related macular degeneration (AMD), which is a major cause of impaired vision in human and retinitis pigmentosa (Ambati et al., 2003; Wright et al., 2010). More recent study conducted on mice deficient for Dicer1 and Dgcr8 in the RPE revealed the presence of abnormalities in photoreceptor maturation including the absence of the photoreceptor outer segments. This observation suggests the non-cell autonomous events directed by miRNAs of RPE in the proper development and morphogenesis of photoreceptor outer segments (Ohana et al., 2015).

1.11 Medaka fish (Oryzias latipes) as model system to study developmental processes

The precise understanding of miRNA function is quite puzzling for several reasons. Often miRNAs are present in clusters and most likely they share a common seed region, which might hinder the information regarding the regulatory mechanisms operated by single miRNA or miRNA cluster. It is more laborious to eliminate all the loci to dissect their global function. Furthermore, each miRNA regulates several targets in different tissues, hence regulates multiple biological events. Target prediction methods consider only the ‘seed’ sequence
compatibility, so it is hard to choose the priority list of putative target genes for the experimental validation. Another important issue is that experimental validation of the possible targets can be cumbersome in mammalian models such as mouse, while in lower vertebrates like medaka fish, it is easy and less time consuming to study the functional roles of each miRNAs in a detailed manner.

Zebrafish and medaka fish are closely related organisms. They separated from their common ancestors about 110 million years ago. Medaka fish has several advantages over other model systems. Medaka fish is very hardy and tolerates a wide range of salinities and temperatures (10–40 °C) and is highly resistant to common fish diseases. In medaka, ovulation is controlled by light. Spawning takes place frequently and there is no limitation in their spawning season. Microinjections to the fertilized egg can be done very easily and it is relatively cheaper. Hence knocking down and overexpression of genes/miRNAs can be easily conducted. Furthermore, generation of knockout and transgenes is less time consuming. Since medaka embryos are transparent, it is a very user-friendly model system to study developmental biology. The genome size of medaka fish is less (800 Mb) compared to zebrafish (1700 Mb). Another advantage of medaka fish over zebrafish is the lower extent of gene duplications in the genome. Medaka fish can attain sexual maturity only in 3-4 months, which is relatively less laborious, time saving and economically less expensive (Wittbrodt et al., 2002). All these features makes medaka fish an easier model organism for developmental and genetics studies.

A large set of information are available for the medaka community at http://www.shigen.nig.ac.jp/medaka/; from which we can share and get the information and reagents. During the past few years this model was extensively used to dissect the regulators
involved in eye development both in physiological and pathological conditions (Alfano et al., 2005; Conte and Bovolenta, 2007; Zhou et al., 2000). Our lab is using medaka fish to dissect the role of miRNAs during various steps of eye development (Avellino et al., 2013; Carrella et al., 2015a; Carrella et al., 2015b; Conte et al., 2010a; Conte et al., 2015). Early embryonic development is slower in medaka fish compared to zebrafish. Zebrafish larvae hatch in 2-3 days, while medaka takes almost 8 days to hatch. Slower development during early phases of development allow easier characterization and identification of developmental stages. The eye development in the medaka starts at the end of gastrulation (stage 15, Fig. 1.9B) with the determination of the eye field. In the late neurula stage (stage 18 Fig.1.9C) the formation of the optic bud (rudimentary eye vesicle) occurs. At stage 21 the optic vesicles differentiate to form optic cups and the early stages of lens formation starts (Fig. 1.9D). At stage 24, the spiracle optical lens is completely formed. (Fig. 9E). At stage 28 retina begins to differentiate and at stage 30 (Fig. 1.9F) plexiform layers start to differentiate. At stage 34 majority of the retinal cell types are formed and retina is well structured (Fig. 1.9G). The eye is completely formed at stage 38 (Fig. 1.9F).

The flexibility of medaka biology, together with the use of sophisticated tools make medaka an attractive model system. This also allowed researchers from different streams of biology to enhance the knowledge including basic cell biological mechanisms to complex drug screening process.
Fig. 1.9. Important stages of medaka fish development:  

A. Stage 1-feretilized egg;  
B. Stage 15-gastrula stage;  
C. Stage 18 –late neurula stage, eye vesicle formation;  
D. Stage 21- 6 somite stage- optic vesicles differentiate to form the optic cups and lens begin to form;  
E. Stage 24- 16 somite stage, the neurocoele is formed in the fore, mid and hind-brains, the formation of spherical lens is completed;  
F. Stage 30- 35 somite stage, the retina start to differentiate;  
G. Stage 34- 5 days, 1hour: majority of the retinal cells are differentiated ;  
H. Stage 38-8 days, hatching stage, eye is completely formed.  
I. 1st fry stage- hatched larva.  

Adapted from (Iwamatsu, 2004)
AIM OF THE PROJECT

This general goal of my work is to gain further insight into the functional role of miRNAs in the development and function of the vertebrate retina. The specific aims of the project are as follows:

1. Identification and functional characterization of novel miRNAs expressed at significant levels in the retina especially in photoreceptors

A group of putatively retina enriched miRNAs were chosen based on a miRNA transcriptome analysis, analysis of their sequence conservation and expression profile analysis by RNA in situ hybridization. Selected miRNAs, which showed expression in photoreceptors were carried forward to do functional studies in the medaka model system by manipulating the miRNA expression levels through gain-of-function and loss-of-function approaches by injecting miRIDIAN Dharmacon microRNA mimics and morpholinos (Mo) (Kloosterman et al., 2007) respectively. The presence of eye developmental defects was monitored during various stages of eye development at morphological and molecular levels.

2. Elucidating the role of miR-204 in photoreceptor differentiation and maturation

In spite of the key regulation of many molecular events mediated by miR-204, until recently nothing was known about the role of miR-204 in photoreceptors (Conte et al., 2015). I used different gain-of-function approaches to explore more on the possible significance of miR-204 in the differentiation and establishment of photoreceptors. I overexpressed miR-204 in both in vitro (661W mouse photoreceptor precursor-like cell line) and in vivo in medaka fish (both by
transient over expression and by characterizing transgenic lines overexpressing miR-204 in different photoreceptor cell types).

3. Identification and functional characterization of miR-204 target genes involved in proliferation and photoreceptor differentiation

A list of predicted target genes of miR-204 which were known to influence the cell proliferation/differentiation were selected based on their expression analysis and literature studies. Among them those which showed the miR-204 binding site conservation in the medaka were chosen to check their expression levels in miR-204 knockdown (by Mo-miR-204 injections) miR-204 overexpression (by injecting miR-204 mimic) medaka models. Finally the genes which were moving in opposite correlation upon overexpression and knockdown of miR-204 were selected for the in vivo validation. Lastly, the putative target genes which showed a phenotypic rescue in the medaka fish were considered as the ‘real targets of miR-204’ in the observed phenotype.

4. Generation and characterization of miR-204 knockout medaka model

Interestingly, in the medaka genome, miR-204 is present in two identical copies in the intronic regions of TRPM1 (Chr. 6) and TRPM3 (Chr. 9) genes. (Conte et al., 2010a). To get wisdom on the expression profile and the functional involvement of each copies of miR-204, I generated and did a preliminary characterization of single miR-204 knockout line (miR-204 located in the intronic region of TRPM3, Chr.9).
5. Characterization of the effect of miR-204 mutation in photoreceptors

MiR-204 is described as a “master regulator” controlling various important molecular events during eye development (Conte et al., 2010a), (Avellino et al., 2013; Conte et al., 2014; Shaham et al., 2013). More recently we have published a pathogenic mutation within miR-204 as the cause of ocular coloboma and retinal dystrophy in human patients (Conte et al., 2015). I was involved in characterizing the consequences of this mutation in photoreceptors by using the medaka model.
2. MATERIALS AND METHODS

2.1 Medaka fish strain and maintenance

*Oryzias latipes* wild type, Cab inbred strain were maintained in an in-house facility with a constant circulating system at 28°C on 14 hours light and 10 hours dark cycle. Embryo stages were calculated according to Iwamatsu 2004 (Iwamatsu, 2004). Embryos were collected by natural spawning and raised in Yamamoto solution at 26°C. All procedures on living animals were performed by anaesthesia administration (tricaine mesylate MS-222, Sigma Aldrich). All fish studies were conducted in strict accordance with the institutional guidelines for animal research and approved by the Italian Ministry of Health.

2.2 Morpholino, mimic and mRNA injections and whole embryo imaging

To inhibit the function of miRNAs, specific morpholinos (Mos) (Gene Tools, LLC) were designed and injected into the fertilized, one-cell stage medaka embryos as previously described (Conte et al., 2010a). Mo-miR-204 used in this study targets both identical copies of the mature miR-204 present in the medaka genome (Conte et al., 2010a). The specificity and inhibitory efficiency and optimal concentrations of Mos were determined on the basis of morphological criteria as described previously (Conte et al., 2010a). For Mo-miR-204, another Mo containing 6 base of mismatch (indicated in red letters below) was used as a control. For miR-210, Mo was designed against mature sequence present in the medaka genome. Target protector morpholino (TP Mo) for Ccnd1 and Cdc44 were designed to block the miR-204 binding region in the 3’ UTR of Ccnd1 and Cdc44. The sequence and concentration of morpholino used in this study are summarized below:
miRIDIAN (Dharmacon) miRNA mimics for miR-204 and miR-210 were injected at a final concentration of 3 μM and 6μM respectively.

The complete coding sequence for the medaka Ccnd1 and Cd44 were obtained from UCSC genome browser and cDNA was synthesised from medaka whole RNA according to manufacturer’s instructions (SuperScript® III First-Strand Synthesis System by Invitrogen). cDNAs were PCR amplified by these set of primers: For Ccnd1- F. primer: GCATCGATAACCTGCTGAACGAGCGGT, R. primer: GCAGATCTTCAAATGTTGATGTCCCGA. For Cd44- F. primer: GCATCGATATGTGGACTTTTCTATTCGG, R. primer: GCAGATCTGAGTGACAGGTGGTCCGAC. The amplicons were cloned in to pCS2 expression vector and transcribed out of pCS2 vector by using SP6 mMessage mMachine kit (Ambion) according to the manufacturer’s instructions. Different concentrations of mRNA were injected in to the medaka
wild type embryos as mentioned earlier. Finally optimum concentration of 10ng/ul was used both for Ccnd1 and Cd44. After the injections, embryo development is monitored carefully and live embryos or post hatching larvae were photographed using Leica stereomicroscope.

2.3 Transformation of E.coli with plasmid DNA

E.coli DH5α cells were grown to mid-log phase (A600=0.6) in Luria Broth (LB: 1% bactotryptone, 1% NaCl and 0.5% Bacto-yeast extract) at 37°C with shaking. Cells were harvested by centrifugation at 2000 x g at 4°C, resuspended into 100ml (for each 100ml of culture) of 50% CaCl2. Suspension was then centrifuged at 5000 x g for 15 min at 4°C. The pellet was resuspended into 100ml (for each 100ml of culture) of 50% CaCl2 and centrifuged again. The cells were resuspended in 3 ml of ice cold 10% glycerol solution, aliquoted and stored at -80°C. For each transformation, DNA was added to 50 µl of competent cells, and incubated in ice for 20-30 minutes then cells were subjected to heat shock at 42°C for 90 seconds and followed by incubation on ice for 5 minutes. Cells were recovered in 1 ml of LB and incubated for 40 minutes to 1 hour at 37°C, before plating on LB-agar containing appropriate antibiotics. Plates were incubated at 37°C overnight to allow bacterial colonies to grow (Sambrook and Russell, 2001). Mini-preps plasmid DNA preparations were carried out using the QIAGEN MINI prep kits. Concentration of plasmid DNA was determined according to the following formula: absorbance of one A260 unit indicates a DNA concentration of 50 µg/ml.

2.4 Whole-Mount In Situ Hybridization

Medaka whole-mount RNA in-situ hybridization was performed, photographed, and sectioned as described previously (Conte and Bovolenta, 2007). The medaka embryos were fixed with 4% paraformaldehyde (PFA) in PTW at room temperature for one hour and shifted to overnight at
4°C with shaking. The embryos were washed with cold 1X PTW and decorionated and finally stored in cold methanol at -20°C at least overnight or until RNA *in situ* hybridization is performed. RNase free reagents were used throughout this experiment. The miRCURY detection probe (Exiqon) for miR-204, miR-210, miR-192 and miR-129 were used according to (Karali et al., 2007). Embryos were gradually rehydrated by washing with 75% methanol/1X PTW, 50% methanol/1X PTW and 25% methanol/1X PTW. Then embryos were treated with 10mg/ml proteinase K in 1X PTW for different period of time depending on their stage (from 5 to 90 minutes). Embryos were washed twice with freshly prepared 2mg/ml glycine in 1X PTW. Embryos were fixed in 4% PFA/PTW at room temperature for 20 minutes and washed 5 times with 1X PTW. Then embryos were pre-hybridised at least for an hour at 65°C (42°C for miRNAs) with hybridisation buffer. The composition of hybridisation buffer is as follows:

- Formamide 100% 25.0ml
- SSC 20X (pH 7.0) 12.5 ml
- Heparin (50mg/ml) 150ul
- Torula-RNA 250 mg
- Tween-20 10%

The probes were added and the vials containing the probe and embryos were incubated at 65°C overnight (42°C for miRNAs). After the incubation embryos were washed at 65°C (42°C for miRNAs) with 50% formamide/2X SSCT, 2XSSCT and 0.2X SSCT respectively. Each washing was for 30 minutes. Then the embryos were incubated at room temperature with a blocking
solution (5% FBS/PTW) for 2 hours with agitation. The embryos were then incubated for 12 hours at 4°C with 200ul of anti-DIG antibody (1:4000 dilution). The embryos were washed 3 times with SB solution (0.1M Tris pH 9.5, 0.1M NaCl, 50mM MgCl₂ and 0.1% Tween-20) and each washing was for 10 minutes. Afterwards NBT/BCIP (Boehringer) was added to the embryos followed by an incubation at 28°C. Once the colour is developed the reaction was stopped by adding TE/0.1% Tween-20. The embryos were again fixed in 4% PFA/PTW for 20 minutes at room temperature, washed with 1X PTW for 3 times and proceeded with the embedding or stored in glycerol until the embedding. The embryos were embedded in proper moulds with a mix of BSA/gelatine and were sectioned with vibratome. The sections were mounted and photographed by using Leica DM-6000 microscopy. Representative Images were taken in bright-field.

2.5 Cell proliferation assay

For the cell proliferation analysis, rabbit α-phospho-histone H3 (PHH3) (1:100; Cell Signaling Technology) and peroxidase-conjugated anti-rabbit antibody (1:200; Vector Laboratories) were used followed by 3, 3-diaminobenzidine staining, as previously described (Beccari et al., 2012). The medaka embryos were fixed with 4% paraformaldehyde (PFA) in PTW at room temperature for one hour and shifted to overnight at 4°C with shaking. The embryos were washed with 1X PTW and decorionated and finally stored in cold acetone at -20°C at least overnight. Then the embryos were washed with 1X PTW for 3 times. Blocking solution (10% FBS in 1X PTW) was added and incubation was done at room temperature for 30 minutes. Rabbit α-phosphohistone H3 (PHH3) (1:100; Cell Signaling Technology) was added to the embryos and incubated at 4°C overnight. Next day the embryos were washed with 1X PTW for 15 minutes, 3 times.
Then peroxidase-conjugated anti-rabbit antibody (1:200; Vector Laboratories) was added followed by an incubation of 2 hours at room temperature. The embryos were washed with 1X PTW for 10 minutes, 3 times. 3, 3-diaminobenzidine (0.67mg/ml of 3,3- diaminobenzidine in 12ul of 30% H₂O₂) was added followed by an incubation in the dark. After the colour development embryos were washed in 1X PTW 3 times and fixed with 4%PFA/PTW for 20 minutes at room temperature. The embryos were washed with 1X PTW 3 times and embedded in to proper moulds by using BSA/Gelatine. The moulds containing samples were sectioned using vibratome. Sections were observed in bright-field with a Leica DM-6000 microscope and PHH3 positive cells were manually counted in the entire Medaka fish retinas.

2.6 Immunofluorescence analysis

Whole medaka embryos were fixed with 4% of paraformaldehyde in PTW, incubated for 1 hour at room temperature and overnight at 4°C with shaking. Embryos were washed 3 times at room temperature with 1X PTW for 10 minutes each time. Embryos were decoreonated, incubated overnight in 15% sucrose/PTW and in 30% sucrose/PTW at 4°C. Embryos were cryopreserved in 50% gelatine and 50% glucose mix. Immunofluorescence was performed on 12uM thick cryosections as described before (Conte et al., 2010a). Sections were washed three times with either 1X PBS (for Zpr-1) or 1X PTW (for Rhodopsin, GS6, Syntaxin, GFP). For each antibody there were different blocking solutions, blocking time, antibody dilutions as summarised below:

<table>
<thead>
<tr>
<th>ANTIBODY NAME</th>
<th>BLOCKING BUFFER USED</th>
<th>CONCENTRATION OF THE ANTIBODY USED</th>
<th>SECONDARY ANTIBODY USED</th>
</tr>
</thead>
</table>

56
<table>
<thead>
<tr>
<th>Protein</th>
<th>Diluent Details</th>
<th>Dilution</th>
<th>Antibody Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhodopsin (Sigma)</td>
<td>10% FBS/1X PTW</td>
<td>1:5000</td>
<td>α-mouse</td>
</tr>
<tr>
<td>Zpr1 (ZIRC)</td>
<td>5% Goat Serum/0.5% Triton X-100/1X PBS</td>
<td>1:500</td>
<td>α-mouse</td>
</tr>
<tr>
<td>GFP (Novus)</td>
<td>3% BSA/5% FBS/0.3% Tween-20/1X PBS</td>
<td>1:500</td>
<td>α-rabbit</td>
</tr>
<tr>
<td>Syntaxin (Invitrogen)</td>
<td>10% FBS/1X PTW</td>
<td>1:100</td>
<td>α-mouse</td>
</tr>
<tr>
<td>GS6 (Invitrogen)</td>
<td>10% FBS/1X PTW</td>
<td>1:100</td>
<td>α-mouse</td>
</tr>
</tbody>
</table>

Slides containing sections were incubated overnight with the primary antibodies then slides were washed three times with 1X PTW followed by an incubation with Alexa Fluor (488 or 594) secondary antibodies (Invitrogen) with 1:1000 dilution. The slides were counterstained with DAPI (Vector Laboratories) and mounted with 50% of Glycerol/1X PBS. Sections were photographed using LSM 700 Zeiss Confocal Microscopy. In case of transgenic lines, the positive anti-Rhodopsin and anti-Zpr-1 cells were counted manually from three consecutive sections with the optic nerve or/and near the optic nerve for each embryo.
2.7 RNA extraction and qRT-PCR

RNA was extracted with DNaseI treatment from the 661W cells and by a pool of embryos or pool of whole eyes of the embryos according to manufacturer’s instructions (QIAGEN miR Nesy mini kit). The cDNAs were prepared by using the Quantitech reverse transcription kit (QIAGEN). The qRT-PCR reactions were done by using the nested primers and carried out with Roche Light Cycler 480 system. The PCR reaction was performed using cDNA (200-500 ng), 10 ul of the SYBR Green Master Mix (ROCHE) and 400 nM primer, in a total volume of 20 ul. The PCR conditions for all the genes were as follows:

<table>
<thead>
<tr>
<th></th>
<th>Temperature</th>
<th>Time</th>
<th>Ramp Rate (°C/s)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Denaturation</strong></td>
<td>95°C</td>
<td>5 min</td>
<td>4.4</td>
<td>1</td>
</tr>
<tr>
<td><strong>Amplification</strong></td>
<td>95°C</td>
<td>10 sec</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60°C</td>
<td>10 sec</td>
<td>2.2</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>15 sec</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td><strong>Melting curves</strong></td>
<td>95°C</td>
<td>5 sec</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>65°C</td>
<td>1 min</td>
<td>2.2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>97°C</td>
<td></td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td><strong>Cooling</strong></td>
<td>40°C</td>
<td>20 sec</td>
<td>1.5</td>
<td>1</td>
</tr>
</tbody>
</table>

Quantification results were expressed in terms of cycle threshold (Ct). The Ct values were averaged for each triplicate. The Hprt and GAPDH genes were used as the endogenous control for all the experiments. Differences between the mean Ct values of the tested genes and those
of the reference gene were calculated as $Ct_{\text{gene}} = Ct_{\text{gene}} - Ct_{\text{reference}}$. Relative expression was analysed as $2^{Ct}$. Relative fold changes in expression levels were determined as $2^{Ct}$ (Alfano et al., 2005).

The sequences of oligonucleotide primers used in this study are summarized below:

<table>
<thead>
<tr>
<th>GENE</th>
<th>PRIMER SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ol-Gapdh-F</td>
<td>5'- CGGCAAGCTGATAGTGATG-3'</td>
</tr>
<tr>
<td>Ol-Gapdh-R</td>
<td>5'- AGAAACACTCCGGTGAGACT-3'</td>
</tr>
<tr>
<td>Ol-Hprt-F</td>
<td>5'- TCTGGAGAGGTTGTACATCC-3'</td>
</tr>
<tr>
<td>Ol-Hprt -R</td>
<td>5'- GATGTAGTCCACAGGTCGG-3'</td>
</tr>
<tr>
<td>Ol-Rhodopsin-F</td>
<td>5'- CATGCACGCTACTTTGTCC-3'</td>
</tr>
<tr>
<td>Ol-Rhodopsin-R</td>
<td>5'- TGATGGGCTTGCAGACAC-3'</td>
</tr>
<tr>
<td>Ol-Zpr-1-F</td>
<td>5'- ATCTGCTCAAGTCTGAGACC-3'</td>
</tr>
<tr>
<td>Ol-Zpr-1-R</td>
<td>5'- TCAGGTCTTGTAGTCCTTC-3'</td>
</tr>
<tr>
<td>Ol-opn1sw-F</td>
<td>5'- CGTTCCTCCATCTCTTTTC-3'</td>
</tr>
<tr>
<td>Ol-opn1sw-R</td>
<td>5'- GAGAACTCTACATCTGCTC-3'</td>
</tr>
<tr>
<td>Ol-opn1mw-F</td>
<td>5'- CCTGGACACTTTCTTTCAG-3'</td>
</tr>
<tr>
<td>Ol-opn1mw-R</td>
<td>5'- TCATTCCCCTGGCATC-3'</td>
</tr>
<tr>
<td>Ol-pre miR-204, chr.6-F</td>
<td>5'- ATGGAGATCCCTCTGTGAC-3'</td>
</tr>
<tr>
<td>Ol-pre miR-204, chr.6-R</td>
<td>5'- TTTTGTGCTCCCTGTATTAC-3'</td>
</tr>
<tr>
<td>Ol-pre miR-204, chr.9-F</td>
<td>5'- CTATGACATGTTGACTCCC-3'</td>
</tr>
<tr>
<td>Ol-pre miR-204, chr.9-R</td>
<td>5'- TGAGCATCCCTTGCCTTC-3'</td>
</tr>
<tr>
<td>Gene</td>
<td>Forward Sequence</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>mmu-Rhodopsin-F</td>
<td>5'-TGTAATCTCGAGGGCTTCTTGG-3'</td>
</tr>
<tr>
<td>mmu-Rhodopsin-R</td>
<td></td>
</tr>
<tr>
<td>mmu-ROM-1-F</td>
<td>5'-GCCATGGCTACAAGGATTGG-3'</td>
</tr>
<tr>
<td>mmu-ROM-1-R</td>
<td></td>
</tr>
<tr>
<td>mmu-Peripherin2-F</td>
<td>5'-CGGGACTGGTCTCGAGATTCC-3'</td>
</tr>
<tr>
<td>mmu-Peripherin2-R</td>
<td></td>
</tr>
<tr>
<td>Ol-Cdh4-F</td>
<td>5'-TCAACATGAAGGCACCGGTC-3'</td>
</tr>
<tr>
<td>Ol-Cdh4-R</td>
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<tr>
<td>Ol-Ccnd1-F</td>
<td>5'-TGAAGGAGACGCTACCATTG-3'</td>
</tr>
<tr>
<td>Ol-Ccnd1-R</td>
<td></td>
</tr>
<tr>
<td>Ol-Ccnd2a-F</td>
<td>5'-TTAAGGGAGACATCGTCTTC-3'</td>
</tr>
<tr>
<td>Ol-Ccnd2a-R</td>
<td></td>
</tr>
<tr>
<td>Ol-Ccnd2b-F</td>
<td>5'-ATTGTCAGGGGCTCCAGAAG-3'</td>
</tr>
<tr>
<td>Ol-Ccnd2b-R</td>
<td></td>
</tr>
<tr>
<td>Ol-Cd44-F</td>
<td>5'-GAAGATGTGTAACAGGTTTG-3'</td>
</tr>
<tr>
<td>Ol-Cd44-R</td>
<td></td>
</tr>
<tr>
<td>Ol-Notch1, chr.9-F</td>
<td>5'-AAACACCCCGTGCAGTCCAC-3'</td>
</tr>
<tr>
<td>Ol-Notch1, chr.9-R</td>
<td></td>
</tr>
<tr>
<td>Ol-Notch1, chr.12-F</td>
<td>5'-TCGATAACCTCCCCTAGTCC-3'</td>
</tr>
<tr>
<td>Ol-Notch1, chr.12-R</td>
<td></td>
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</tbody>
</table>

Ol-medaka, mmu-mouse
To detect the mature form of miRNA taqman qRT-PCR was performed as described previously (Carrella et al., 2015a). The cDNAs for the Taqman were prepared with the Taqman miRNA reverse transcription kit (Applied Biosystems) by using 10ng of RNA. The qRT-PCR reactions were performed with Roche Light Cycler 480 by using Taqman miRNA assays by Applied Biosystems. For the medaka experiments custom made O.l.U6-snRNA with the following sequence:

AGTTCTCTAGTCATCTACTAAATTGGAACGATACAGAGAAGATTAGCATGGCCCTGCCTCGCAAGGATGA
CACGCAAATTCGTGAAGCGTTCC

and for the 661W cell line RNU48 were used as endogenous controls. A reaction of 20ul was prepared in triplicates. The PCR conditions are mentioned below:

<table>
<thead>
<tr>
<th></th>
<th>Temperature</th>
<th>Time</th>
<th>Ramp.Rate</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre-incubation</strong></td>
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<tr>
<td><strong>Amplification</strong></td>
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<td>4.4</td>
<td>45</td>
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<tr>
<td></td>
<td>60°C</td>
<td>1 min</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td><strong>Cooling</strong></td>
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<td>30 sec</td>
<td>2</td>
<td>1</td>
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</tbody>
</table>

The Ct values were averaged for each in-plate technical triplicate. The averaged Ct was normalized as difference in Ct values (ΔCt) between the miRNA in analysis and endogenous controls to each sample in analysis. Then the ΔCt values of each sample were normalized with respect to the ΔCt values of the control (ΔΔCt). The variation was reported as fold change (2-ΔΔCt).
2.8 661W cell differentiation, transfections and cell cycle analysis

661W cells were cultured and differentiated as described previously (Meola et al., 2012) with slight modifications. Cells were grown in 10 ml dish at 37°C and 5% CO₂ in DMEM low glucose(1g/L) medium supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (50 mg/mL). The cells were trypsinised and plated in 6 well plates upon 100% confluency. After 12 hours the cells were transfected with miR-204 mimic by using INTERFERin Transfection Reagent according to manufacturer’s instructions (Polyplus-transfection), miRIDIAN Dharmacon negative mimic was transfected as control. Differentiating medium was added to the cells 24 hours after the transfection. RNA was isolated at different time points (T0, T6, T12, T18, T24) during the differentiation as described before.

The cell cycle analysis was done by staining the cells with Propidium Iodide (PI) as described before (Meola et al., 2012). The cells were trypsinised, washed with cold DPBS and incubated in 70% Ethanol at 4C overnight. The cells were washed again with DPBS and treated with 100ng/ml of RNAse and then stained with 20ug/ml PI, incubated on ice in dark for at least 30 minutes. The PI stained sample were read by using BD Accuri C6 Flow Cytometer. The percentage of cells in different phases of the cell cycles were measured by using BD Accuri C6 software.

Unless mentioned elsewhere, each *in vitro* experiment was performed using three biological replicates.
2.9 Generation of transgenic medaka line

A 3.2-kb TαC promoter fragment replicates the temporal and spatial pattern of endogenous TαC expression in zebrafish (Kennedy et al., 2007). The conservation of this regulatory region in medaka fish was confirmed by UCSC Genome Browser (https://genome.ucsc.edu). 3.2 Kb of the medaka TαC regulatory region was amplified by using this couple of primers: F. primer- GTGGATCCCTGATAGGAAAATAATGGAG, R. primer- CTATCGATTTCTGGCCTATGTTGATTAG and cloned into the pSKII-ISceI-eGFP vector. The promoter sequence was placed upstream to miR-204 precursor sequence present within human β-globin intron. The purified construct (10ng/ul DNA in ISce I enzyme reaction) was injected in to the fertilized one cell stage of medaka embryos as described previously (Conte and Bovolenta, 2007). The embryos were selected based on the GFP expression in the eyes observed under Leica UV flurescent stereo microscopy, raised to sexual maturity and transgenic founder fish were identified for further breeding.

2.10 Generation of TALEN mediated miR-204 knockout line

A pair of TALEN was used to induce targeted mutagenesis in the intronic region of TRPM3 gene where one of the copies of miR-204 is located. Custom-designed TALEN vectors were assembled by ZGenebio (ZGenebio Biotech Inc, Taiwan). The left (L) and right (R) recognition sequences and the spacer sequence used were: L- CACCTTACTATGACAT (16 bp); R- GATTCCAGGCATAGGAT (17 bp); spacer sequence GTGGACTTTCCCTTTGTC (17 bp). The TALEN RNA was obtained by transcription according to the manufacture’s instructions (mMESSAGE mMACHINE SP6 Transcription Kit, Ambion, Life Technologies) and injected into fertilized eggs at 1-cell stage. After hatching, larvae were allowed to grown to maturity. G0 fish were genotyped by using the genomic DNA derived from the caudal fin. The genomic DNA was isolated and the
region containing miR-204 in TRPM3 was amplified by using this couple of primers: F. primer: AAGCTCCCTTGTAACATTG, R. primer: TCATATCCAACCGATTTG. PCR products were sequenced in PrimBiotech. Sequencing results were read using Chromas Lite software. G0 fish with specific mutation were selected and mated with wild-type (wt) fish to obtain heterozygous animals. G1 heterozygous fishes bearing the desired mutation were selected and mated to generate a knockout line of miR-204.

2.11 In silico analysis

Publicly available expression databases storing the information on miRNA expression by RNA in situ hybridization, such as Geisha (chicken expression database, http://geisha.arizona.edu/geisha/), EUREXpress (mouse embryo database, http://www.eurexpress.org/ee/) and miRNeye (mouse embryo and adult, http://mirnemye.tigem.it) were used to understand the exact expression of miRNAs selected from miRNome (small RNA seq). Conservation of the miRNAs and its target sites across the species were checked by using miRBase (http://www.mirbase.org/) and UCSC genome browser (https://genome.ucsc.edu/). Bioinformatic tools such as TargetSCAN (http://www.targetscan.org/), PicTar (http://pictar.mdc-berlin.de/), miRanda (http://www.microrna.org/), starBase (http://starbase.sysu.edu.cn/) were used to predict the possible target genes of miR-204. Enrichment analysis of the selected putative targets of miR-204 were done using DAVID functional annotation tool (https://david.ncifcrf.gov/).

2.12 Statistical analysis

A paired two-tailed T-test was carried out to test the variation of a single factor between two variables (two samples/treatments). A GLM (Generalised Linear Model) was performed to
understand the response variables that have error distribution models other than a normal
distribution (number of photoreceptors in the retina deriving from different animals, number of
cells in a particular phase of cell-cycle). Two-way ANOVA was performed to test the variation of
more than one factor among multiple variables (multiple samples/treatments). In all the cases
p-value ≤ 0.05 was considered as statistically significant.
3. RESULTS

3.1 Functional characterization of novel miRNAs expressed in retina especially in photoreceptors.

3.1.1 Selection criteria of the novel miRNAs

In order to identify new miRNAs, which are highly expressed in retina especially in photoreceptors, I took advantage of a miRNome (small RNA seq) analysis that was performed on human retina in my lab (Karali et al., 2016). To identify specific retina-enriched miRNAs, I compared the list of expressed miRNAs in retina with the miRNAs expression profile of non-ocular cell types, such as HeLa cells. Interestingly, this analysis yielded a total of 15 novel putative retina-enriched miRNAs. I also verified the expression profile of the selected miRNAs in other model organisms such as chicken and mouse. To do this, I used publicly available expression databases storing information on miRNA expression by RNA in situ hybridization, such as Geisha (chicken expression database, http://geisha.arizona.edu/geisha/), EURExpress (mouse embryo database, http://www.eurexpress.org/ee/) and miRNeye (mouse embryo and adult, http://mirneye.tigem.it). After analysing all the publicly available miRNAs expression databases, I found a total of 9 miRNAs putatively expressed in retina at significant levels out of 15 selected miRNAs. I further selected miRNAs that were found to be strongly conserved across evolution, particularly in medaka fish. Out of the 9 miRNAs selected from the previous step, 5 were conserved in medaka fish.
Since I was interested in novel miRNAs, I excluded the miRNAs, which were already partially characterized by other groups. As a final result, I selected for further characterization three miRNAs, namely miR-210, miR-192, and miR-129, which are conserved in the medaka (Fig. 3.1).

**3.1.2 Expression analysis of the selected miRNAs**

To determine and compare the precise expression profiles of selected miRNAs, I used whole mount RNA *in situ* hybridization (ISH) analysis on post-hatching medaka larvae (stage 40) where the retina is fully differentiated and functional. Interestingly, expression of these miRNAs was found in different retinal cell types. The expression of miR-210 was found in retinal ganglion cells, amacrine cells and in photoreceptors (Fig. 3.2A&B). Expression of miR-129 was evident in...
amacrine cells and in the RPE (Fig. 3.2A) whereas expression of miR-192 was restricted to the RPE (Fig. 3.2A). Based on the above data, due to the fact that miR-210 was expressed in photoreceptors, I selected miR-210 for further investigation.

3.1.3 Gain and loss of function analysis of miR-210

In order to understand the possible role of miR-210 in medaka retina development and function, I interfered the miR-210 function by both loss-of-function (by Mo-based knockdown approach) and gain-of-function (by miRNA mimics injection) assays in wild-type medaka embryos at one cell stage. To this end, I designed a Mo against the miR-210 mature sequence present in the medaka genome. I set-up and injected a wide range of Mo (0.05mM, 0.1mM, 0.2mM, 0.3mM and 0.4mM) and miRNA mimic (1uM, 2uM, 3uM, 4uM, 5uM and 6uM) concentrations. 200 embryos were injected and analysed either with morpholino or with mimic at each different concentrations mentioned (Fig. 3.3B). Interestingly, embryos showed a

Fig 3.2. Expression analysis of the selected miRNAs in medaka fish by RNA in situ hybridization. (A) miR-210 is expressed in ganglion cells, amacrine cells and in photoreceptors. miR-129 is expressed in amacrine cells and in RPE. miR-192 is expressed in RPE. RNA in situ hybridisation was performed at stage 40. Arrows indicate the localization of miRNA expression. (B) Enlarged miR-210 expression profile. GCL- ganglion cell layer; INL- inner nuclear layer; ONL- outer nuclear layer; RPE- retinal pigment epithelium.
developmental delay during the early stage of development (stage 28) with the highest injected concentration of both morpholino and miRNA mimic, i.e., 0.4mM and 6uM respectively (Fig. 3.3A). This delay in the development of the injected medaka embryos was not any more evident at later stages (after stage 34). Notably, embryos fully recovered this developmental delay during later stages of development. To further investigate on a possible defect of the retina, due to miR-210 alterations, I sectioned and analysed the eyes of the embryos injected by

Fig 3.3. Gain and loss of function studies of miR-210 at earlier stages. (A) Maximum concentrations of morpholino and miRNA mimic injected embryos showed delay in development during early stages of development (stage 28). Arrows indicate the underdeveloped parts. (B) Table summarising the various concentrations of the morpholino and miRNA mimic injected and the number of embryos analysed in each concentration of injection.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Morpholino</th>
<th>miRNA mimic</th>
</tr>
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<tbody>
<tr>
<td>0.05mM, 0.1mM, 0.2mM, 0.3mM, 0.4mM</td>
<td>1uM, 2uM, 3uM, 4uM, 5uM, 6uM</td>
<td></td>
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<tr>
<td>Embryos analysed in each concentration</td>
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</table>
Mo-miR-210 and mimic miR-210 at stage 40. However, I did not observe any defects in retinal layering by DAPI staining and morphological analysis (Fig. 3.4A). In addition, I did not observe any qualitative phenotypic differences in photoreceptor cells (following the injection of both Mo and miRNA mimic) by performing immunofluorescence analysis for Rhodopsin, a rod photoreceptor specific marker (Fig. 3.4B). Since morphological and preliminary molecular analysis did not give any ocular specific phenotype, I decided not to pursue further studies on miR-210.

3.2. Elucidating the role of miR-204 in photoreceptor differentiation and maturation

3.2.1 Transient overexpression of miR-204 in medaka fish:

In the recent past years, our lab has contributed comprehensively to understand the different roles played by miR-204 in different ocular cell types at various steps of eye development. (Avellino et al., 2013; Conte et al., 2010a; Conte et al., 2015; Conte et al., 2014). Even though the role of miR-204 in regulation of multiple events during eye development has been described in various retinal cell types, its role in the photoreceptor cells has not been elucidated so far with the exception of our recent publication wherein we have identified and demonstrated that a mutation within miR-204 was a cause of ocular coloboma with retinal dystrophy and photoreceptor degeneration in human patients (Conte et al., 2015). To test the functional role of miR-204 in photoreceptors, I used a gain-of-function approach. Therefore, I transiently overexpressed miR-204 in medaka embryos by injecting miR-204 mimic as described in the materials and methods section. The miR-204 mimic-injected embryos showed a microphthalmia phenotype as described before. Notably, microphthalmia observed here upon
overexpression of miR-204 was milder compared to the one observed in miR-204 morphants (Conte et al., 2010a; Conte et al., 2015). To investigate further on this phenotype and to understand the possible contribution of miR-204 in cell proliferation/differentiation, I carried out Phospho-Histone H3 (PHH3) staining on retina (Fig. 3.5A). PHH3 is a marker for the cells undergoing mitosis (Colman et al., 2006). I noticed that upon PHH3 staining the eyes of the embryos overexpressing miR-204 showed a lower numbers of mitotic cells with respect to control eyes at stage 24 and 30 (Fig. 3.5B). To get deeper insight into the contributions of miR-

**Fig 3.4. Phenotypic characterization of gain and loss of function of miR-210 at later stages of development.** (A) The images of the control, morpholino and miRNA mimic injected embryos at stage 40. The eyes were sectioned and stained with DAPI. No detectable phenotype was observed in the layering of retina upon knockdown or overexpression of miR-210. (B) Immunofluorescence for Rhodopsin at stage 40 with control, morpholino and Mimic miR-210 injected embryos. Apparently there was no morphologically visible ocular phenotype.
204 to photoreceptor homeostasis, I further performed RNA in situ hybridization for the rod specific gene *Rhodopsin* at stage 34, a stage in which Rhodopsin expression can be detected at the protein level (Kitambi and Malicki, 2008) and at stage 36. Interestingly, I found an earlier and increased expression of the Rhodopsin signal at stage 34 and at stage 36 (Fig. 3.6A) in the embryos injected with the miR-204 mimic. Also, by qRT-PCR we found increased levels of Rhodopsin transcripts (Fig. 3.6B). Moreover immunofluorescence for Rhodopsin and Zpr-1 at stage 34 revealed the earlier expression of photoreceptor markers upon overexpression of

![Image of P-H3 staining and graphs](image)

**Fig 3.5. Effect of miR-204 on mitosis.** (A) Phospho Histone H3 (PHH3) staining in control and miR-204 mimic injected embryos at stage 30. Less number of PHH3 cells can be noticed in miR-204 overexpressing retina compared to control retina. (B) Graph representing the number of positive cells for PHH3 staining at different stages (st. 24, 30, 34) of embryo development. Increase number of PHH3 positive cells were found at stage 24 and stage 30 in miR-204 mimic injected emryos than in control. At stage 34 no significant difference in the number of PHH3 positive cells was detected.
miR-204 (Fig. 3.7A). With the immunofluorescence for anti-Rhodopsin and anti-Zpr-1 I could not appreciate any difference in the expression of both Rhodopsin and Zpr-1 at later stages of development (stage. 38). However the distribution of both the markers remain unaltered in both control and miR-204 overexpressing embryos (Fig. 3.7A). The Rhodopsin transcript was found upregulated at stage 38 as shown by qRT-PCR analysis (Fig. 3.7B). Overall, transient overexpression of miR-204 in the medaka embryos showed to play a possible role in early exit of the retinal cells most probably photoreceptor precursors and also this observation indicate the role of miR-204 in either ‘photoreceptor differentiation’ or in ‘photoreceptor cell fate determination’.

3.2.2 Generation and characterization of transgenic lines overexpressing miR-204 in different photoreceptor cell types

In the present study, transient over expression of miR-204 has some limitations such as dilution of injected mimic at later stages of embryonic development. To overcome the dilution effect of miRNA mimic and to better understand the roles exerted by miR-204 exclusively in photoreceptors, I generated and characterized stable transgenic lines, in which miR-204 was expressed under the control of promoters of genes which are specifically expressed exclusively in cones or in rods. In the medaka fish the photoreceptor differentiation and development starts at later stages during the embryo development (Kitambi and Malicki, 2008). First, I generated and characterized a transgenic line expressing miR-204 under the control of TαC a cone specific promoter. The miR-204 promoter was placed between the human β-globin intron to ensure the splicing; GFP was placed downstream to miR-204 precursor (Fig. 3.8A).
Fig 3.6. Effect of miR-204 on the differentiation of photoreceptors (I). (A) Representative images of RNA in situ hybridization for Rhodopsin at stage 34 and stage 36. Rhodopsin expression was visible earlier in miR-204 mimic injected embryos compared to controls as indicated by red boxes. Also Rhodopsin expression is covering larger part of retina in miR-204 mimic injected embryos. (B) qRT-PCR analysis showed a significant upregulation Rhodopsin transcript levels at stage 34 and stage 36.
The medaka embryos were injected with this construct along with \textit{l-sceI} meganuclease enzyme. Co-injection of \textit{l-SceI} meganuclease with a reporter construct flanked by \textit{l-SceI} site helps earlier transgene integration into the host genome (Soroldoni et al., 2009). The cone specific transgenic line was named as TαC:GFP:miR-204. Transgenic embryos showed GFP expression in the eye (cones) starting from stage 32 when observed under fluorescent stereomicroscopy. Embryos, starting from stage 32 were sectioned and stained with DAPI to visualise the nuclei and the sections were seen under the LSM 700 confocal microscopy in order to confirm that the
GFP expression is localized exclusively in cones (Fig. 3.8B). By Taqman qRT-PCR (by using RNA extracted by pool of whole eyes) at stage 32 and at stage 40, miR-204 levels were found to be increased by around 50% confirming the overexpression of miR-204 in TαC:GFP:miR-204 transgenic line (Fig. 3.8C). In the medaka fish the first mature cones can be found at around
stage 32 (Kitambi and Malicki, 2008). To further investigate on the effect of miR-204 overexpression only in cones, I began with the immunofluorescence for Zpr-1 at early stages of cone development (stage 32 and stage 34). Remarkably, at both the stages, I found an increase in the expression of Zpr-1 markers covering a wider area in the TαC: GFP: miR-204 embryos compared to wild type (Fig. 3.9A). To understand the precise number of Zpr-1 positive cells both in TαC:GFP:miR-204 and in wild type embryos, I counted the number of Zpr-1 positive cells in three consecutive sections close to the optic nerve or/and with the optic nerve from 7 different embryos at stage 32 and 6 different embryos at stage 34 and I noticed increase in the number of Zpr-1 positive cells in the TαC:GFP:miR-204 embryos than in control embryos (Fig. 3.9B&C). These results clearly show the impact of miR-204 in cone photoreceptor maturation.

Since rods differentiate and attain maturation much later than cones (Valen et al., 2016), I was eager to know the ‘non-cell autonomous effect’ on rods in the cone specific transgenic line (TαC:GFP:miR-204). In the medaka fish Rhodopsin expression can be detectable around stage 34 onwards (Kitambi and Malicki, 2008). Therefore, I did the immunofluorescence analysis for Rhodopsin at early stages of rod development i.e., at stage 34 and at stage 36 to appreciate if there was any alteration in the expression of Rhodopsin (Fig. 3.10A). In order to get an exact number of cells stained with the anti-Rhodopsin antibody, I counted the number of Rhodopsin positive cells manually under LSM 700 confocal microscopy at 40X in three consecutive sections near the optic nerve or/and with the optic nerve in 6 different medaka embryos at stage 34 and 7 different embryos at stage 36 and the results were represented graphically (Fig. 3.10 B&C). At stage 34 there is a trend of increased number of Rhodopsin positive cells even though the difference is not statistically significant (Fig. 3.10B). At stage 36 there were some Rhodopsin
positive cells found in the ventral part of the retina in the transgenic embryos whereas wild type embryos did not show this trend (Fig. 3.10A). With the graph it is very much evident that the number of Rhodopsin-positive cells in the transgenic embryos (TαC:GFP:miR-204) was
significantly higher compared to wild type embryos (Fig. 3.10C). The above results indicate a possible ‘non-cell autonomous ‘effect of miR-204 on rods, indicating that the overexpression of miR-204 in cones exert an impact either in the acceleration of ‘rod photoreceptor cell fate specification’ or earlier/increased rod photoreceptor maturation.

To know in depth whether the overexpression of miR-204 in the cone photoreceptors modulates the distribution of Zpr-1 and Rhodopsin markers at later stages of development, I performed immunofluorescence for Zpr-1 at stage 36 and immunofluorescence for Rhodopsin and Zpr-1 at stage 38 (Fig. 3.11A). There was no visible difference in the distribution of the above mentioned markers in both the stages tested. In order to understand the levels of Rhodopsin and Zpr-1 expression levels, I checked the levels of transcripts of the wild type and transgenic (TαC:GFP:miR-204) embryos at stage 36. I found a significant increase in the level of Zpr-1 transcript and there was also a trend of increase in the transcript level of Rhodopsin, S-opsin (opn1sw) and M-opsin (opn1mw) (Fig. 3.11B). To investigate further on the molecular networks mediated by miR-204 over expression in photoreceptors, I took advantage of a previously generated transgenic line over expressing miR-204 exclusively in rod photoreceptor cells. More specifically, this transgenic line was named Rho:TK:GFP:miR-204, in which miR-204 precursor was under the control of the rhodopsin promoter, miR-204 precursor was inserted within the human B-globin intron in the 5’UTR of the GFP gene reporter (Fig. 3.12A). The expression of the GFP reporter gene allowed me to select the embryos overexpressing the miR-204. To verify the expression of miR-204 in rod photoreceptor cells, I carried out a preliminary screening by sectioning the eyes of embryos (which were screened for GFP expression in the eye) starting from stage 34, 36, 38 and 40 and staining them with DAPI. Then sections were
photographed with LSM 700 confocal microscope to confirm the GFP expression only in the rods (Fig. 3.12B). I also performed the Taqman qRT-PCR on total RNA extracted from the eyes of Rho:TK:GFP:miR-204 embryos at stage 40. Notably, I found a two fold increase in the level of expression of the miR-204 in Rho:TK:GFP:miR-204 transgenic embryos compared to wild type

Fig 3.10. Over expression of miR-204 in cones leads to an increase in the no. of rods. (A) Representative images with the immunofluorescence for Rhodopsin at stage 34 and stage 36. The Rhodopsin positive cells are indicated by arrows. (B&C) Graph representing the no. of Rhodopsin positive cells in control and TaC:GFP:miR-204 eyes at stage 34 and at stage 36. Embryos of TaC:GFP:miR-204 showed significantly higher no. s of Rhodopsin positive cells than controls.
embryos (Fig. 3.12C). I also performed RNA in situ hybridization in order to understand the expression of miR-204 in Rho:TK:GFP:miR-204 transgenic embryos. Also RNA in situ hybridization for miR-204 clearly showed the intense expression of miR-204 in the outer nuclear layer of Rho:TK:GFP:miR-204 retina in comparison with wild type retina at stage 40 (Fig. 3.12D). I started characterizing this transgenic line (Rho:TK:GFP:miR-204) firstly by doing

Fig 3.11. Overexpression of miR-204 in cones did not alter the distribution of photoreceptor markers at later stages of development. (A) Representative images from immunostaining of Zpr-1 at stage 36, 38 and immunostaining for Rhodopsin at stage 38 from TαC:GFP:miR-204 embryos showed no alteration in the distribution of these markers. (B) qRT-PCR analysis at stage 36 indicated significant increased levels of Zpr-1 transcripts and also there was a trend of increased levels of Rhodopsin, opn1sw and opn1mw transcripts in TαC:GFP:miR-204 line compared to control.
immunofluorescence analysis for rods (anti-Rhodopsin staining) at stage 34 and stage 36 (Fig. 3.13A). Manual counting of the anti-Rhodopsin positive cells from the sections with or near the optic nerve allowed me to have an absolute number of anti-Rhodopsin positive cells or mature rods both in the wild type and in the Rho:TK:GFP:miR-204 transgenic embryos. There was an
increase in the numbers of anti-Rhodopsin positive cells or mature rods at stage 34 and stage 36 in Rho:TK:GFP:miR-204 transgenic line in comparison with wild type (Fig. 3.13B&C).

In order to understand the ‘non-cell autonomous’ consequences of over expression of miR-204 in rods on the other photoreceptor cell type i.e., cones, I did the immunofluorescence experiments against Zpr-1 at stage 36 (Fig. 3.14A) and counted the number of anti-Zpr-1 stained cells. I found no significant difference in the number of anti-Zpr-1 positive cells in the transgenic

Fig 3.13. Overexpression of miR-204 in rod photoreceptors leads to an earlier increase in the number of rod photoreceptors. (A) Representative images with the immunofluorescence for Rhodopsin at stage 34 and 36. The Rhodopsin positive cells are indicated by arrows. (B&C) Graphs representing the no.s of anti-Rhodopsin positive cells in control and Rho:TK:GFP:miR-204 retina at stage 34 and stage 36. Rho:TK:GFP:miR-204 retinas showed significantly higher no. of anti-Rhodopsin positive cells at stage 36. There was a trend of increase in the no. of anti-Rhodopsin positive cells were found at stage 34.
(Rho:TK:GFP:miR-204) embryos with respect to wild type embryos (Fig. 3.14B). As mentioned before, cone photoreceptor differentiation and maturation happens in advance than rod photoreceptors. Therefore overexpression of miR-204 in the rods might not have any effect in modulating the cone ‘fate determination’ or cone maturation.

To understand the effect of miR-204 overexpression in rods at later stages during development, I performed immunofluorescence analysis of Rhodopsin and Zpr-1 at stage 38 and at stage 40. Notably, I found no evident difference in the distribution of both cone and rod specific markers in Rho:TK:GFP:miR-204 embryos in comparison with wild type (Fig. 3.15). Overall, miR-204 exerts a role in rod maturation in the transgenic line Rho:TK:GFP:miR-204 but no effect on the cones were observed.
I speculated whether the overexpression of miR-204 in photoreceptors (cones and rods) could perturb the homeostasis of other retinal cell types. To address this, I did some preliminary experiments such as immunofluorescence for GS6 (Muller glia cell marker) and Syntaxin (a marker for amacrine cell and ganglion cell synaptic terminals). I noticed no observable difference in the expression of these markers in both TαC:GFP:miR-204 and Rho:TK:GFP:miR-204 transgenic embryos with control embryos (Fig. 3.16).

Fig 3.15. Effect of overexpression of miR-204 in rods at later stages of photoreceptor development. Representative images of immunostaining for Rhodopsin and Zpr-1 at stage 38 and 40 from Rho:TK:GFP:miR-204 and control retinas. There was no evident difference in the distribution of two markers tested in Rho:TK:GFP:miR-204 and control retinas.
3.2.3 Characterization of miR-204 function in 661W mouse photoreceptor precursor-like cells

I decided to use a very convenient in vitro model system to perceive further on the miR-204 mediated regulations of molecular events in photoreceptor cells in a more elaborated way. To that purpose I chose to use the 661W cell line, which is a mouse retinoblastoma cell line overexpressing the SV40T antigen under the control of the IRBP (intra retinal binding protein) promoter (al-Ubaidi et al., 1992). Previous studies on this cell line have shown that it can

Fig 3.16. Overexpression of miR-204 in cones or rods did not alter the distribution of other retinal markers. Immunofluorescence images for GS6 at stage 40 and Syntaxin at stage 36 showed no detectable change in the distribution/expression of GS6 and Syntaxin markers in both TαC:GFP:miR-204 and Rho:TK:GFP:miR-204 transgenic lines compared to controls.
differentiate towards a photoreceptor-like cell fate upon addition of a specific differentiating medium and one can also see the expression of some of the photoreceptor specific markers (Meola et al., 2012; Tan et al., 2004). Primarily, I monitored the level of miR-204 at different time points (T0, T6, T12, T18, T24) during the differentiation process of 661W by using Taqman qRT-PCR. Interestingly, there was an increase in the endogenous levels of miR-204 at ‘T12’ compared to control. Starting from ‘T18’, levels of miR-204 went down (Fig. 3.17A). This result again suggests the salient role of miR-204 during the differentiation of 661W cells. I further investigated the importance of miR-204 in 661W cell line by overexpressing miR-204 specifically by transfecting miR-204 mimic and miRNA negative mimic was used as a control. Differentiating medium was added 24 hours after the transfection of miR-204 or control. Cell samples were collected at different time intervals during the differentiation (T0, T6, T12, T18, T24). From one set of cell samples, cell cycle analysis was performed by staining the cells with PI, which is a nucleic acid interacting agent and a fluorescent molecule. Then sorting was done depending upon the DNA content present in the cell by flow cytometer. Interestingly, at ‘T12’, a higher percentage of cells were found in the G0/G1 phase (62.44%) in miR-204 transfected samples compared to control samples (59.25%) and there was a lower percentage of cells G2/M phase (29.84%) in miR-204 transfected cell samples compared to controls (32.98%) (Fig 3.18A&B). From the other set of the collected cell samples whole RNA was isolated and the transcript levels of different photoreceptor markers were checked by qRT-PCR. I found that there was an earlier expression and increased levels of photoreceptor markers like Rhodopsin, ROM1 and Peripherin2 in the miR-204 transfected cell samples compared to control cell samples (Fig. 3.17B). The peak of expression was found at ‘T18’ of the differentiation and at
Fig 3.17. Characterization of the effect of miR-204 in 661W cell line. (A) Taqman qRT-PCR revealed the expression of miR-204 during different time points of differentiation of 661W cells towards a photoreceptor like cell fate. The highest level of miR-204 was detected at 12 hours of differentiation (T12) and at later time points expression levels of miR-204 went down. (B) qRT-PCR analysis for Rhodopsin, ROM1 and Periferin2 markers showed an upregulation of these marker levels earlier during differentiation in miR-204 mimic (miR-204 OE) transfected samples compared to control samples. Highest level of expression was found at T18 during differentiation. After T18 expression levels of these markers went down.
later stages expression of the above mentioned markers started to go down in the cells transfected with miR-204. On the other hand, in the control cell samples, most of the markers were still upregulated at ‘T24’ (Fig. 3.17B) Overall, the above mentioned results on 661W cell line provides further evidence to the role of miR-204 in ‘earlier fate determination’ and/or maturation of photoreceptors and also its role in the cell cycle alteration.

3.3 Identification and functional characterization of miR-204 target genes involved in proliferation and photoreceptor differentiation

3.3.1 Analysis of miR-204 targets involved in cell proliferation and differentiation

Identifying the target gene(s) of a miRNA in a given mechanism/phenotype of interest is one of the significant steps while studying functional consequences of miRNAs in any biological process. Overexpression of miR-204 both in medaka fish and in 661W cell line altered the cell cycle and differentiation/cell fate determination of photoreceptor cells. I hypothesised that the observed phenotype is because of the perturbation of putative target gene(s) of miR-204, involved in cell cycle regulation or differentiation. It was an important task to identify and dissect the molecular mechanisms involved in the phenotype observed. I took advantage of some bioinformatics tools that allow to predict a list of possible target genes for miRNAs. Tools such as TargetSCAN (http://www.targetscan.org/), PicTar (http://pictar.mdc-berlin.de/), miRanda (http://www.microrna.org/), starBase (http://starbase.sysu.edu.cn/) predict the interaction between a microRNA and an mRNA target. By taking advantage of the above mentioned tools, I selected a list of predicted targets of miR-204. Then I did the enrichment analysis of the selected genes by taking advantage of DAVID functional annotation tool
Fig 3.18. Role of miR-204 in altering cell cycle in 661W cell line. (A) Cell cycle analysis with Propidium Iodide (PI) staining resulted in more percentage of cells in G0/G1 phase and less percentage of cells in G2/M phase in miR-204 OE samples with respect to control at T12. In the other time points no significant difference in the percentage of cells at different cycling phase were observed in miR-204 overexpressing (OE) samples and in controls. (B) Table summarising the percentage of cells at G0/G1 phase, S-phase and G2/M-phase during ‘T12’ both in control and miR-204 OE samples.
I selected a subset of 20 genes enriched in biological processes such as cell cycle and neuronal differentiation. Then I did literature searches and binding site conservation analysis in medaka with the selected subset of 20 genes. Finally I selected 5 putative target genes of miR-204 (Cdh4, Ccnd1, Ccnd2, Cd44 and Notch1), which might be the possible candidate genes that could have a role in the phenotype of my interest.

In medaka Ccnd2 and Notch1 are present in two different copies. So I used different set of primers to amplify each copy of a single gene. I checked the transcript levels of the selected putative target genes in miR-204 knockdown (by injecting morpholino) and miR-204 overexpressing (by injecting miRNA mimic) medaka models. I extracted RNA at stage 30 of embryo development, this was the stage in which we found the alteration in the number of mitotic cells upon PHH3 staining previously. qRT–PCR revealed the expression levels of all the putative targets of miR-204 tested. Among them Ccnd1 and Cd44 were moving in opposite correlation upon knockdown and over expression of miR-204 (Fig. 3.19B&C). Therefore I selected Ccnd1 and Cd44 for further validation process.

**3.3.2 In vivo validation of the putative target genes**

I used the medaka in vivo system to validate whether Ccnd1 and Cd44 were the ‘real targets’ of miR-204 involved in the microphthalmic phenotype upon miR-204 overexpression. To understand this I took the advantage of target protector morpholino (TP Mo) approach. Target protector morpholinos are antisense oligonucleotides of 25-30 bp in length, which can bind perfectly to the site in the 3’UTR that is complementary to the miRNAs of the target gene from miRNA (Staton and Giraldez, 2011).
To check the above mentioned hypothesis of TP Mos, I injected different concentrations of TP Mo-Ccnd1 and TP Mo-Cd44 to the medaka embryos at one cell stage. A concentration above 0.09mM led to embryo lethality at later stages of development. At 0.09mM of TP Mo injection, I found the eye phenotype similar to the one of miR-204 knockdown characterised by severe microphthalmia, coloboma and lens herniations (Fig. 3.20A). As mentioned before, miR-204 overexpression by miRNA mimic injection led to milder form of microphthalmia. As an evidence to our hypothesis, co-injected miR-204 and TP Mo-Ccnd1/TP Mo-Cd44 miR-204 were able to rescue the microphthalmic phenotype in around 80% of the embryos injected (Fig. 3.20B&C).
Fig 3.20. In vivo validation of putative target genes (I). (A) Representative images showing the severity of the ocular phenotype in TP Mo-Ccnd1 and TP Mo-Cd44 injected embryos. Injected embryos showed severe microphthalmia and microcephaly as indicated by red lines. (B) Injection of mimic miR-204 (miR-204) alone showed mild microphthalmic phenotype, whereas co-injection of miR-204 and TP Mo-Cd44 was able to rescue the microphthalmia. (C) Injection of mimic miR-204 (miR-204) alone showed mild microphthalmic phenotype, co-injection of miR-204 and TP Mo-Ccnd1 was able to rescue the microphthalmic eye phenotype. (D) Immunofluorescence analysis of anti-Rhodopsin upon injection of miR-204 showed earlier/increased expression of Rhodopsin marker. Co-injection of miR-204 and TP Mo-Ccnd1 was able to retain the expression of Rhodopsin to normal level. Localization of Rhodopsin marker is indicated by arrows.
At the molecular level, I found that the expression of Rhodopsin was restored after the co-injection of miR-204 mimic and TP Mo-Ccnd1 at stage 34 (Fig. 3.20D). I also hypothesised that by co-injecting the Ccnd1 and Cd44 coding region (cds) along with miR-204 should restore the phenotype if the phenotype is due to the low dose of Ccnd1 and/or Cd44. Indeed, I found that co-injection of miR-204 mimic along with the full length Ccnd1 and Cd44 mRNAs in medaka embryos were able to rescue the microphthalmic phenotype (Fig. 3.21A&B). The above results suggest the involvement of Ccnd1 and Cd44 in the microphthalmic phenotype and consequent earlier expression of photoreceptor markers, most likely by direct targeting of miR-204.

### 3.4 Generation and characterization of miR-204 knockout medaka model

During recent past years our lab has contributed comprehensively to understand the different roles played by miR-204 in different ocular cell types at various steps of eye development. (Avellino et al., 2013; Conte et al., 2010a; Conte et al., 2015; Conte et al., 2014). Interestingly, miR-204 is present in two identical copies in the medaka genome. One of the copies of miR-204 (miR-204.1) is located in the intronic region of TRPM1 gene, chromosome 6 and the other copy of miR-204 (miR-204.2) is located in the intronic region of the TRPM3 gene, chromosome 9 (Conte et al., 2010a). In medaka, TRPM3, one of the host genes of miR-204 is expressed in the RPE, inner nuclear layer and ganglion cell layer (Fig. 3.22). Another host gene of miR-204 TRPM1 is expressed in the outer nuclear layer, in the optic nerve, lens and in the ciliary marginal zone (Fig. 3.22). Whereas miR-204 is expressed highly in the RPE, lens, ganglion cell layer and in the inner nuclear layer (Fig. 3.22).
In the previous studies conducted in our lab, we have used a morpholino-mediated approach to knockdown miR-204 to study the effect of miR-204 ablation in the development of medaka embryos. However, morpholinos act by inhibiting both copies of miR-204 and Mos are diluted in cells upon embryo development. To disentangle the contribution of either copy of miR-204 and to generate stable miR-204 KO medaka line I used a TALEN based technology. The transcription activator like effector nuclease (TALEN) technology has become a powerful approach for genome editing in a number of animal models (Sander et al., 2011).

Fig 3.21. In vivo validation of putative target genes (II). A&B Images of miR-204 injected and cds ccnd1+miR-204 and cds cd44+miR-204 injected embryos. Upon co-injection of cds ccnd1, miR-204 and cds cd44, miR-204 rescue of microphthalmic phenotype was found. The size of the eye is indicated by red lines.
TALENs consist of a fusion between a FokI nuclease domain and a transcription activator like (TAL) effector DNA recognition domain, which cuts the double stranded DNA at user specific positions. The cut ends will re-join by homologous end joining or non-homologous recombination hence inducing different mutations in the region of interest (Cermak et al., 2011) (Fig. 3.23A). I used specific TALENs in order to target one of the copies of miR-204, i.e., miR-204.2 located within the intronic region of \textit{TRPM3} gene. I used a left TALEN conjugated with GFP (Green Fluorescent Protein) and a right TALEN conjugated with Red Fluorescent Protein (RFP) (Fig. 3.23B). In this way I could select the embryos injected by observing them under a fluorescent stereomicroscope. I selected the embryos, which have both GFP and RFP expression and that could therefore be chimera for miR-204 mutations. I selected G0 fish for a particular mutation and mated with wild-type (wt) fish to obtain heterozygous animals. Then G1 heterozygous fish bearing the desired mutation were selected and mated to generate

![Fig 3.22. Expression profile of miR-204 and its host genes. Images of in situ hybridization and RNA in situ hybridization performed at stage 38 for TRPM3, TRPM1 and miR-204. TRPM3 is expressed in RPE-Retinal Pigmented Epithelium; INL-Inner Nuclear Layer; GCL-Ganglion Cell Layer. TRPM1 is expressed in ONL-Outer Nuclear Layer; near the optic nerve, lens and ciliary marginal zone. Expression of mir-204 was found in the RPE-Retinal Pigmented Epithelium; near the optic nerve, INL-Inner Nuclear Layer; GCL-Ganglion Cell Layer; lens and near the ciliary marginal zone.](image)
Fig 3.23. **Working strategy of TALENs.** (A) Working strategy of TALENs. (B) TALEN constructs maps used to generate miR-204.2 knockout medaka line. pZGB-3L corresponds to left TALEN construct and conjugated with EGFP and pZGB-3R corresponds to right TALEN construct and conjugated with mCherry.
a knockout line of miR-204. The mutations obtained were different deletions and substitutions in the ‘seed’ region of miR-204. Among them, the most frequent mutations were deletions of a base (n.20ΔT), three bases (n.20_22ΔTCC), five bases (n.20_24ΔTCCCT) and deletion of five bases along with a substitution of one base (n.20_24ΔTCCCT,n.25T>G) in the ‘seed’ regions (Fig. 3.24A). In all the mutant embryos, the precursor levels of pre-miR-204 (TRPM3, Chr.9) were downregulated to a great extent and the host gene TRPM3 levels were unaltered (in most of the mutants) (Fig. 3.24B). The expression level of mature form of miR-204 was reduced approximately to half in all the mutants (Fig. 3.24C) suggesting the complete depletion of miR-204.2. Further characterization of miR-204.2 knockout line needs to be done in order to understand the contribution of one of the copies of miR-204 in ocular development and function.

3.5 Characterization of the effect of miR-204 mutation in photoreceptors

MiR-204 is described as a “master regulator” controlling various important molecular events during eye development (Conte et al., 2010a),(Avellino et al., 2013; Conte et al., 2014; Shaham et al., 2013). As mentioned before, recently we have identified a mutation n.37C > T, in the seed region of miR-204 was resulted in ocular coloboma along with retinal dystrophy including photoreceptor degeneration in human patients. We have demonstrated that the mutation was likely acting in a gain-of-function manner (Conte et al., 2015). I was involved partly in the in vivo studies of the mutant miR-204 (n.37C > T), specifically focusing on the photoreceptors.

When we overexpressed miR-204 n.37C>T in medaka, we found the impairment of the eyecup morphology. At stage 38 microphthalmia and coloboma were evident in the mutants in comparison with control (Fig. 3.25A&B). These phenotypic alterations were different and more
**Fig 3.24. Characterization of miR-204.2 knockout line.** (A) Sequence of WT and other mutants obtained by TALEN technology. ‘Seed’ region of miR-204 is marked by red letters. Deletion of different nucleotide is marked as ‘–’ and substitution of nucleotide is indicated by green letter. (B) qRT-PCR analysis for miR-204.2 precursor and TRPM3 gene in different mutants. Levels of the precursors are reduced in all the mutants. (C) Taqman qRT-PCR analysis to detect the mature form of miR-204 in different mutant embryos. In all the mutants levels of the mature miR-204 is reduced around 50%.
severe than those observed after overexpression of the wt-miR-204 mimic (Fig. 3.25A&C). To evaluate whether the n.37C > T variation also had an effect on photoreceptors, we analysed the retinas of mut-miR-204–injected embryos at St. 38 by doing the immunofluorescence for Rhodopsin and Zpr-1. Throughout the entire retina, we observed a notable reduction of both Rhodopsin and Zpr-1 markers mut-miR-204 overexpression in comparison with both wt-miR-204 and control injected embryos. (Fig. 3.25D-I). Overall, these results strongly suggest the importance of miR-204 in photoreceptor development.
Fig 3.25. Characterization of the effect of miR-204 mutation in photoreceptors. (A–C) Bright-field microscopy images of lateral views eyes from control-injected (A), mut-miR-204–injected (B), and wt-miR-204–injected (C) embryos. (D–E) Representative frontal eye sections immunostained with an anti-Rhodopsin (green) and Zpr-1 (G–I; green) antibodies, from st38 control-injected (D and G), mut-miR-204–injected (E and H), and wt-miR-204–injected (F and I).
4. DISCUSSION

I started my project with an aim to disentangle the functional roles of retina enriched miRNAs by studying individual miRNA functions during retina development. Part of my study was largely focused on elucidating the functional roles of miR-204 in photoreceptors.

I began by analysing the miRNA transcriptome (miRNome) of human retina performed in our lab (Karali et al., 2016). MiR-210 was one of the selected and most promising miRNAs chosen. The expression of miR-210 was detected in inner nuclear layer, ganglion cell layer and in photoreceptor (Fig 3.2A&B). MiR-210 has been known to regulate tissue development, tumorigenesis and post-ischemia (Biswa et al., 2010; Huang et al., 2009). Overexpression of miR-210 in non-neuronal cells impairs proliferation (He et al., 2013; Tsuchiya et al., 2011; Zhang et al., 2009). Furthermore, gene ontology analysis predicted and verified mRNA targets of miR-210 reveals that many are involved in cell cycle regulation and, perhaps, in neural development (Fasanaro et al., 2009). One of the findings reported by Abdullah and colleagues, reveals the role of miR-210 in neural progenitor (NP) cell-cycle progression. Overexpression of miR-210 promotes premature cell-cycle exit and terminal differentiation in NPs whereas downregulation of miR-210 promotes an increase in the radial glial cell population and delayed differentiation, which leads to increase in the number of late born postmitotic neurons (Abdullah et al., 2016).

Surprisingly, in my case, despite the fact that, miR-210 expression was appreciable in many neuronal layers of developing retina, its gain-of-function and loss-of-function in medaka did not yield any detectable eye phenotype at morphological level (Fig. 3.4A&B). Yet, the majority of the embryos (75%) injected with Mo-miR-210 and mimic miR-210 showed general
developmental delay only during early stages (stage 28-30) of development (Fig. 3.3A). But at the later stages of development (starting from stage 32 onwards) retinal architecture and entire body of the embryos at morphological level were normal (Fig. 3.4A). Since the developmental delay I observed during the embryo development did not last longer, it indicates the probability of alteration in the cell cycle progression during early developmental stages due to the manipulation of miR-210 expression as explained by (Abdullah et al., 2016).

Another miRNA of great interest during ocular development is miR-204. Our lab has contributed extensively in understanding the different regulatory mechanisms played by miR-204 in different ocular cell types during various stages of development. MiR-204 has very distinct expression pattern in the eye during development. Its expression is detectable in the RPE, neural retina, lens and the ciliary body in the fish, mouse and human (Conte et al., 2010a; Deo et al., 2006; Karali et al., 2010; Karali et al., 2007; Shaham et al., 2013; Wang et al., 2010; Xu et al., 2007). A large set of evidence points out that miR-204 plays an essential role in the differentiation and function of all the ocular cell types in which it is expressed (Adijanto et al., 2012; Avellino et al., 2013; Conte et al., 2010a; Conte et al., 2014; Shaham et al., 2013; Wang et al., 2010). Until recently, the role of miR-204 in the photoreceptors was unknown (Conte et al., 2015). My study started with an aim to dissect the functional part of miR-204 in photoreceptors.

In the present study, I described for the first time to my knowledge, the consequences of miR-204 overexpression on photoreceptors. In medaka, transient overexpression of miR-204 (by injecting mimic miR-204) resulted in a mild microphthalmic phenotype (Fig. 3.21A). The molecular phenotype of the miR-204 overexpressing embryos were characterized by a lower
PHH3 positive cells at early stages of embryo development (Fig. 3.5A&B) and earlier and increased expression of Rhodopsin and Zpr-1 markers (Fig. 3.6A, B, 3.7A). Similarly, miR-204 gain-of-function studies conducted on 661W, a mouse photoreceptor precursor-like cell line also showed the alteration in the cell cycle (Fig. 3.18A&B) and earlier expression of photoreceptor markers such as Rhodopsin, ROM1 and Peripherin2 (Fig. 3.17B). Several lineage studies have showed that the retinal progenitor cells remain uncommitted to a specific cell fate until the time of the final cell cycle (Goetz et al., 2014; Turner and Cepko, 1987; Turner et al., 1990). Although exceptions to this have been reported (Ezzeddine et al., 1997; Malicki, 2004).

In the present study, since there was an alteration in the cell cycle (both in vivo and in vitro) and earlier increased expression of Rhodopsin and other photoreceptor markers, it is suggesting the direct link between the affected cell cycle and earlier expression of photoreceptor markers or in other words earlier differentiation of photoreceptors is due to the consequence of earlier exit of the precursors from the cell cycle. The expression of miR-204 during the differentiation of 661W (Fig. 3.17A) towards a photoreceptor-like cell fate strengthen the presumption that miR-204 is necessary during the differentiation. Another study states the role of miR-204/211 in the differentiation of human fetal RPE (hfRPE) cells. According to the findings of this study, levels of miR-204/211 were upregulated during differentiation of hfRPE, whereas downregulation of both the miRNAs was noticed during dedifferentiation (Adijanto et al., 2012). Moreover, according to a study conducted by Ohana and colleagues, miR-204 regulates differentiation program in RPE and it also necessary for the maturation of photoreceptors.(Ohana et al., 2015). These findings underline the importance of miR-204 in photoreceptors and also adds an additional value to the role of miR-204 in the differentiation process.
Several studies explain the influence of various intrinsic and extrinsic factors on the ‘cell fate determination’ and differentiation of retinal neurons, reviewed in (Cepko, 2014; Stenkamp, 2015). Otx2 (orthodenticle homeobox 2 protein) is a key transcription factor necessary for the photoreceptor development. The early expression of Otx2 in the progenitors helps the cells to acquire photoreceptor and bipolar cell identity. Crx (cone rod homeobox) gene is present downstream to Otx2 and is required for the accurate expression of almost all photoreceptor genes (Chen et al., 1997; Freund et al., 1997; Furukawa et al., 1997). Depletion of Crx leads to severe reduction in expression levels of photoreceptor specific genes (Samuel et al., 2014). Other studies conducted on the transcription factors like Ascl1 (Mash1), Foxn4, Rorβ, Atoh7 (Math5), Neurod1, Thrb2 (Thrb), Olig2 indicate the prominence of these factors in molecular mechanisms of photoreceptor development/differentiation, reviewed in (Brzezinski and Reh, 2015; Wang and Cepko, 2016). Another possible explanation to earlier expression of Rhodopsin and other photoreceptor specific markers in the miR-204 overexpression models could be because of the interplay of any of the transcription factors mentioned above. This theory looks convincing because of a reason that at later stages and time points during the differentiation process the level of most of the photoreceptor markers starts to go down or remain highly unchanged, while in the control samples, the expression of these markers were still shooting up (Fig. 3.17B) meaning that the early increase in the levels of photoreceptor markers is an indication of the early onset of differentiation. Once the photoreceptors are completely differentiated, there is no further increase in the level of markers. Yet, there was an upregulation in the transcript levels of Rhodopsin at stage 38 in miR-204 overexpression medaka model (Fig. 3.7B).
Some reports in literature suggest that during ‘fate determination process’ influence of some signalling molecules and transcription factors favour the differentiation of one neuronal cell type over the other most likely with the expense of early-born cell type. Otx2 has been reported to activate another transcription factor Prdm1 (Blimp1) in a direct manner (Brzezinski et al., 2010; Wang et al., 2014). Prdm1 prevents Otx2 expressing cells from attaining bipolar cell fate. In Prdm1 mutants, Otx2 expressing cells begin to develop to get photoreceptor identity but later switch to bipolar fate (Brzezinski et al., 2010; Brzezinski et al., 2013; Katoh et al., 2010). Preliminary findings of my study indicates the absence of any alteration in the distribution of other retinal markers like for Muller glia cells (anti-GS6), Amacrine cells and synaptic terminals of ganglion cells (anti-Syntaxin) as shown by immunofluorescence experiments in miR-204 OE models (Conte et al., 2015). However, milder form of microphthalmic phenotype observed upon miR-204 overexpression strongly suggests the lack in numbers of one or more types of ocular cells. Further research on this aspect can reveal the happenings in the entire retina. Importantly, miR-204 overexpression did not lead to cell death in the retina at different stages tested (stage 24, 30, 34) (Conte et al., 2015).

To get more insight into the role of miR-204 exclusively in photoreceptors, I also used stable transgenic lines expressing miR-204 under the control of promoters of genes, which are specifically expressed in cones and in rods. Cone transducin α (TαC) is a cone specific gene expressed in cone photoreceptors. In zebrafish, the expression of TαC was localized with the expression of cone specific markers like Zpr-1 and UV opsins but not with the Rhodopsin (Kennedy et al., 2007). In zebrafish, regulatory region of TαC was used to direct the transgene expression of wild type TαC in achromatopsia nof mutants (Kennedy et al., 2007). Interestingly,
in medaka, overexpression of miR-204 exclusively in cone photoreceptors (TαC:GFP:miR-204) resulted in an increase in the number of Zpr-1 positive cells or, in other words, in the number of cone photoreceptors starting from stage 32, a stage in which first mature cones are visible (Fig. 3.9A-C). This observation suggests that, in this case, miR-204 is not making an impact towards the differentiation of cones because the TαC gene is expressed not in cone precursors but rather in developed cones. The reason for the observed phenotype could be (i) either early born cone photoreceptors in which miR-204 is overexpressed may be influencing the genesis of other cone precursors to differentiate towards ‘cone fate’ or (ii) somehow first born cone photoreceptors are accelerating the maturation of other cone photoreceptors which are already committed towards ‘cone fate’. Interestingly, in this transgenic line, I also found increase in the number of Rhodopsin positive cells starting from stage 34, a stage in which mature rods are visible. At stage 36 the increase in the number of rods is significant (Fig. 3.10A-C), suggesting the possibility of non-cell autonomous impact of miR-204 on rods likely by affecting the differentiation of rod photoreceptors because the rod photoreceptors are formed later during the development compared to cones. In the rod specific transgenic line Rho:TK:GFP:miR:204, miR-204 was placed downstream to Rhodopsin promoter. The number of Rhodopsin positive cells were found more at early stages of rod differentiation (Fig. 3.13A-C) but the number of the Zpr-1 positive cells or the number of cones are un-altered in this transgenic embryos compared to wild type (Fig. 3.14A&B) as expected.

Previous studies done on the photoreceptor differentiation and genesis have highlighted some signalling molecules and transcription factors, which play a major role in this process. Otx2 expression is one of the earlier signs of photoreceptor transition. Inhibition of Notch signalling
causes the expression of Otx2 in the most progenitors in mouse retina (Jadhav et al., 2006; Nelson et al., 2006; Yaron et al., 2006) while conditional deletion of Notch pathway components reduced the number of progenitors and increases the number of rod and cone photoreceptor precursors (Jadhav et al., 2006; Mizeracka et al., 2013; Riesenberg et al., 2009; Yaron et al., 2006). Additionally, another study conducted on mice RPC (retinal progenitor cells) discloses the varying expression of a gene Olig2 across time. When the daughters of E13.5 to E14.5 Olig2 expressing RPCs were clonally labelled by retroviral infection, only cones and horizontal cells were marked. When day P0 or P3 Olig2-expressing RPCs were marked by viral infection, only rods and amacrine cells were labelled. The RPC clones lacking Olig2 were comprised of rods and bipolar cells, as well as rods and Muller glial cells (Wang and Cepko, 2016). If the overexpression of miR-204 in cones/rods is affecting any factors or/and the components of any pathways discussed above, then it is very much expected that the number of photoreceptors will vary in a large manner. It is also important to mention that combined regulatory activity of several RPE rich miRNAs families such as miR-204/211, miR-222/221, miR-20b/106a have been reported to have an impact on the maturation of photoreceptors (Ohana et al., 2015).

At later stages of photoreceptor development starting from stage 36, compact arrangement of the photoreceptors limit the precise counting of cells stained with specific antibody. However, in TαC:GFP:miR-204 transgenic line the levels of transcripts of Rhodopsin, Zpr-1 and opsins showed increased trend (Fig. 3.11B) but in both transgenic lines the distribution of both Rhodopsin and Zpr-1 markers remain unaltered in the transgenic embryos compared to control embryos (Fig. 3.11A&15) perhaps indicating the absence of photoreceptor cell death. Literature
studies reveal the involvement of several miRNAs in the maintenance of mature photoreceptors. In mice, conditional knockout of DICER1 in rods leads to progressive, early-onset retinal degeneration and functional impairment (Sundermeier et al., 2014). MiR-182 and miR-183 are necessary for the maintenance of mature cone photoreceptor outer segments (Busskamp et al., 2014). However, precise investigations are required in the future to understand the role of miR-204 at later stages of photoreceptor development. Additionally, preliminary experiments suggest that there is no alteration in the distribution of other retinal markers like GS6 and Syntaxin in both the transgenic embryos in comparison with controls (Fig. 3.16) indicating the absence of non-cell autonomous impact of miR-204 on the other retinal cells.

After analysing all the data from both the transgenic lines, it appears that there are same/similar molecular mechanism of action or the pathways and molecular regulators are involved in both cone and rod specific transgenic lines. Overall, it is more acceptable by the set of evidences given by current work that miR-204 plays a role in maturation of both cone and rod photoreceptors. Further efforts should be made to uncover the molecular events in both TαC:GFP:miR-204 and Rho:TK:GFP:miR-204 transgenic lines.

Another possible phenomenon occurs during the cell cycle which could impact highly on the cell fate specification is the activation of cell cycle-inhibitors like CDKIs or down regulation of cell-cycle activators such as cyclins and CDKs. Retinoblastoma (Rb) protein promotes cell cycle exit by inhibiting cell division and suppresses re-entry of differentiated cells back to cell cycle (Burkhart and Sage, 2008). The phosphorylation status of Rb during the cell cycle depends mainly upon CDKs, which can function by binding to cyclin proteins (Dyer and Cepko, 2001).
One of the major cyclins in the retinal development is *Ccnd1*, which binds to CDK4/6. *Ccnd1* is highly expressed globally in retinal progenitor cells but after differentiation its levels goes down (Barton and Levine, 2008; Dyer and Cepko, 2001; Sicinski et al., 1995). *Ccnd1* contributes to two aspects of proliferation by controlling the rate of cell cycle progression and the timing of cell cycle exit (Das et al., 2009). Interestingly, I found that levels of *Ccnd1* were moving in opposite correlation to each other upon overexpression and downregulation of miR-204 (Fig. 3.19B&C). This observation can be explained by previous studies conducted on *Ccnd1*, which explains the loss of *Ccnd1* in mice results in severe microphthalmia because of the reduced proliferation of retinal progenitor cells (Das et al., 2009; Fantl et al., 1995). Moreover, the cell cycle is prolonged, premature exit of the RPCs resulted in differentiation defects showing a higher proportion of RGCs and photoreceptors at the expense of horizontal and amacrine cells (Cunningham et al., 2002). Most of the observations made previously with respect to cell cycle are similar to the observations made in my study. Since I have noticed a lower number of cells in mitosis (Fig. 3.5A&B) and a higher percentage of cells at G0/G1 phase (Fig. 18A&B) upon miR-204 overexpression, one could argue that this is most likely due to the pre-mature cell cycle exit resulting out of down regulation of *Ccnd1*. I also observed microphthalmic phenotype in miR-204 overexpression medaka embryos, however the form of microphthalmia is of milder degree. As mentioned earlier, except photoreceptors, apparently other retinal cells are not affected however this needs further confirmations. According to another study, knockdown of *Ccnd1* expression resulted in microphthalmia with no severe defects in differentiation process (Duffy et al., 2005). Experimental data of my research are promising that *Ccnd1* is the direct target of miR-204 in the phenotype I observed. Inhibition of miR-204 binding to the target site in *Ccnd1*
by injecting morpholino target protector (TP Mo) showed severe microphthalmia similar to that of miR-204 morphants (Fig. 3.20A). Whereas co-injection of mimic miR-204 and TP Mo-Ccnd1 showed rescue of the microphthalmic phenotype and also showed normal Rhodopsin expression (Fig. 3.20C&D). Moreover, overexpression of Ccnd1 (by injecting the mRNA of Ccnd1) was able to rescue the microphthalmic phenotype (Fig. 3.21A). All these observations strongly infer that Ccnd1 has a role in phenotype observed in miR-204 overexpressing models. Hence promising to be the direct target of miR-204 in the phenotype observed.

It is well known fact that miRNAs can target multiple genes. Along with Ccnd1, another transcript Cd44 was also moving in opposite trend upon overexpression and downregulation of miR-204 (Fig. 3.19B&C). Cd44 (cluster of differentiation 44) antigen is a surface glycoprotein with a single polypeptide chain, encoded by the Cd44 gene (Tammi et al., 2008; Thapa and Wilson, 2016). Embryos injected with TP Mo-Cd44 alone showed severe microphthalmia (Fig. 3.20A), similar phenotype was observed before in the miR-204 morphants. Co-injection of miR-204 mimic and TP Mo-Cd44 was able to rescue the milder form of microphthalmia observed in miR-204 overexpressing embryos (Fig. 3.20B). Like in case of Ccnd1, Cd44 full length mRNA and miR-204 mimic co-injections were able to restore the microphthalmia (milder) phenotype observed upon miR-204 overexpression (Fig. 3.21B). Almost certainly, miR-204 is targeting both Ccnd1 and Cd44 in the miR-204 overexpressing models and hence Ccnd1 and Cd44 are contributing towards microphthalmic phenotype, premature exit of the cell cycle and ultimately to the early onset of photoreceptor differentiation. Further in vitro evidences such as luciferase assay are needed to add additional weightage to the rescue experiments involving TP-Mos.
Accumulating evidence during the past few years suggest that mutation in the miRNA or its target genes may result in to human pathogenicity including ocular disorders (Iliff et al., 2012; Lechner et al., 2013; Mencia et al., 2009; Solda et al., 2012). In the recent past, our lab took part in identification of a dominant mutation in miR-204 as a genetic cause of retinal degeneration and ocular coloboma in a British family. We have demonstrated that this mutation is acting likely through a gain-of-function manner (Conte et al., 2015). I was part of in vivo characterization of this mutation in medaka. I mainly focused on the consequences of this mutation in photoreceptors. Interestingly, overexpression of mut-miR-204 resulted in severe eye abnormalities including severe microphthalmia and optic coloboma compared to controls (Fig. 3.25A-C). Photoreceptor abnormalities were evident at later stages characterized by loss of photoreceptor markers (Fig. 3.25D-I). These results clearly support the deleterious effects played by mutant form of miR-204.

In lower vertebrates such as medaka fish, miR-204 is present in two identical copies (Conte et al., 2010a). In medaka, considering the fact that miR-204 is present in two identical copies, it is important to understand the expression pattern and roles played by each copies of miR-204 in the retina. In this regard, I generated single knockout line for one of the copies of miR-204 (miR-204.2) present in the intronic region of TRPM3 gene. As mentioned in the results part of this thesis, the mutations are characterised by various deletions and substitutions in the seed part of miR-204 (Fig. 3.24A). In all the mutations the mature miR-204 level was reduced to around 50% (Fig. 3.24C) indicating the complete loss of one of the mature forms of miR-204. Generation of the knockout medaka line for another copy of miR-204.1 (located in TRPM1) is needed in future. The detail characterization of both single and double knockout of miR-204
may help to address several curious questions regarding the subtle roles played by each copies and it might also help to understand the evolutionary significance of miR-204/211 to a larger extent.

In summary, findings of this study suggests an important role of miR-204 in photoreceptor differentiation and maturation and provides another evidence to the contribution of this miRNA in ocular development.
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6. REFERENCES


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MiR-204 is responsible for inherited retinal dystrophy associated with ocular coloboma


The eye is a major target tissue for genetic disease. The main group of genetic disorders affecting the retina is represented by inherited retinal dystrophies that include, among others, retinitis pigmentosa, one of the leading causes of inherited blindness in the Western population, with a prevalence of 1/4,000 (1). The loss of vision in severe retinal dystrophies is most often a result of progressive loss or dysfunction of photoreceptor cells or retinal pigment epithelial (RPE) cells. In addition to degenerative disorders, the eye is also the target of a number of developmental genetic disorders, among which the group classified as microphthalmia, anophthalmia, and coloboma (MAC) features major structural eye malformations. Both inherited retinal dystrophies and MACs are characterized by extreme genetic heterogeneity. According to recent estimates, more than 150 retinal dystrophy genes (www.omim.org) have been reported to date, including several that account for conditions in both groups. Many others remain to be identified for both conditions.

Ocular developmental disorders, including the group classified as microphthalmia, anophthalmia, and coloboma (MAC) and inherited retinal dystrophies, collectively represent leading causes of hereditary blindness. Characterized by extreme genetic and clinical heterogeneity, the separate groups share many common genetic causes, in particular relating to pathways controlling retinal and retinal pigment epithelial maintenance. To understand these shared pathways and delineate the overlap between these groups, we investigated the genetic cause of an autosomal dominantly inherited condition of retinal dystrophy and bilateral coloboma, present in varying degrees in a large, five-generation family. By linkage analysis and exome sequencing, we identified a previously undescribed heterozygous mutation, n.37C>T, in the seed region of microRNA-204 (miR-204), which segregates with the disease in all affected individuals. We demonstrated that this mutation determines significant alterations of miR-204 targeting capabilities via in vitro assays, including transcriptome analysis. In vivo injection, in medaka fish (Oryzias latipes), of the mutated miR-204 caused a phenotype consistent with that observed in the family, including photoreceptor alterations with reduced numbers of both cones and rods as a result of increased apoptosis, thereby confirming the pathogenic effect of the n.37C>T mutation. Finally, knockdown assays in medaka fish demonstrated that miR-204 is necessary for normal photoreceptor function. Overall, these data highlight the importance of miR-204 in the regulation of ocular development and maintenance and provide the first evidence, to our knowledge, of its contribution to eye disease, likely through a gain-of-function mechanism.

MicroRNAs (miRNAs) are key regulators in the regulation of gene expression. An understanding of human conditions caused by microRNA mutations provides insight into mechanisms of gene regulation and into the interplay between development and maintenance in tissue homeostasis. The eye represents a notable target tissue of genetic diseases. Inherited retinal degenerations and developmental eye disorders are two separate groups that represent leading causes of blindness worldwide. Identifying underlying genetic causes of such conditions is important for diagnosis, counseling, and potential therapy development. We identified a dominant mutation in miRNA-204 as the genetic cause of a unique phenotype of retinal degeneration and coloboma and thus highlight the importance of microRNA-204 as a master regulator of ocular development and normal maintenance.

Significance

MicroRNAs are key players in the regulation of gene expression. An understanding of human conditions caused by microRNA mutations provides insight into mechanisms of gene regulation and into the interplay between development and maintenance in tissue homeostasis. The eye represents a notable target tissue of genetic diseases. Inherited retinal degenerations and developmental eye disorders are two separate groups that represent leading causes of blindness worldwide. Identifying underlying genetic causes of such conditions is important for diagnosis, counseling, and potential therapy development. We identified a dominant mutation in microRNA-204 as the genetic cause of a unique phenotype of retinal degeneration and coloboma and thus highlight the importance of microRNA-204 as a master regulator of ocular development and normal maintenance.

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individuals of a five-generation family of white British descent. Family history suggested an autosomal dominant pattern of inheritance, and full clinical examination demonstrated that the phenotype was not associated with extraocular manifestations.

Individual V-1 was diagnosed with retinal dystrophy and bilateral iris coloboma in early childhood. At age 18 y, he had reduced visual acuities (0.74 LogMar right, 0.84 LogMar left) and was registered as partially sighted. Mild hypermetropic astigmatism was observed (right eye, $+1.75/+0.50 \times 100$; left eye, $+2.25/0.50 \times 85$). Color vision was significantly compromised bilaterally in the City University color vision test (third edition, 1998) and the Ishihara color vision screening test (38-plate edition, 1979). Bilateral iris colobomata with iridolenticular adhesions was present with no evidence of cataract (Fig. 1B). Dilated

Fig. 1. Ocular phenotype of family with inherited retinal dystrophy and iris coloboma. (A) Pedigree of the family. The proband is denoted by an arrow. *, patients affected with retinal dystrophy and coloboma who underwent exome sequencing; ●, all patients for whom DNA was available for testing. (B and C) Slit lamp biomicroscopy photographs of the left eye of affected family members are presented; a high degree of interocular symmetry was observed. The left eye of patient V-1 (B) revealed inferior iris coloboma (black arrow) and iridolenticular adhesions (green arrow). The left eye (C) of patient IV-1 demonstrated inferior iris coloboma. (D) OCT (Heidelberg Spectralis) of the central macula of the right eye of patient V-1 is shown (Top); a high degree of interocular symmetry was observed, and an image from the right eye of an unaffected individual is also shown for comparison (Bottom). The OCT suggests extensive loss of the hyporeflective band corresponding to the photoreceptor nuclei (red arrow). (E) Electroretinography of left (Left) and right (Right) eye of patient V-1 is shown. The black lines correspond to the recorded traces and the gray areas represent the normal limits. Extinguished responses to all conditions are observed except for a minimal light-adapted 30-Hz flicker response corresponding to residual cone function.
posterior segment examination revealed scattered RPE mottling with retinal atrophy and attenuation of the retinal vasculature. Optical coherence tomography imaging was consistent with severe photoreceptor loss (Fig. 1D). Electroretinography revealed extinguished right and left eye responses to all conditions except a minimal residual light-adapted 30-Hz flicker response (Fig. 1E); notably, 4 y before, electrodiagnostic testing had revealed less attenuated light-adapted responses. The proband’s mother, IV-I, aged 42 y, presented with a similar phenotype, having been diagnosed with bilateral iris colobomata and a progressive retinopathy in infancy. She underwent bilateral cataract surgery at age 30 y and subsequently had a, likely iatrogenic, retinal detachment in her left eye. She was registered as blind at age 30 y and at age 42 y has hand movement vision in her right eye and no perception of light in her left eye. Bilateral iris colobomata are noted (Fig. 1C). Individuals II-1, III-1, III-2, and III-3 were all registered blind. All had bilateral iris coloboma and a slowly progressive retinal dystrophy leading to marked loss of vision in late childhood or early adult life. Individual III-2 was also found to have congenital cataracts and underwent right cataract surgery at age 7 y.

Identification of a miR-204 Mutation by Linkage Analysis and Exome Sequencing. To identify possible chromosomal regions associated with the retinal dystrophy and iris coloboma phenotype in this family, parametric linkage analysis was performed for six individuals with the phenotype and parental origin of transmission was determined. Using linkage analysis, a region on 19q13 was implicated as a possible chromosomal region associated with the phenotype. Exome sequencing was performed on all available family members and the proband. The novel miR-204 mutation (n.37C>T) identified in a family with autosomal dominant retinal dystrophy and coloboma. (A) Electropherograms showing the 22-nt mature sequence of mir-204. Patient sequence (Top) contains the heterozygous mutation (n.37C>T), which segregated among affected individuals and the wt sequence (Bottom) observed in unaffected individuals. The boxed sequence indicates the 7-bp seed region. (B) Multiple species alignment showing segment of the mir-204 sequence, including the 5p arm (shaded area), obtained using CLUSTALW software. The human mutation at nucleotide position +37 is indicated in red, and the underlined sequence denotes the seed region. bta, Bos taurus; dre, Danio rerio; fru, Fugu rubripes; gac, Gasterosteus aculeatus; gga, Gallus gallus; ggo, Gorilla gorilla; Hsa, Homo sapiens; mdo, Monodelphis domestica; mmu, Mus musculus; ola, Oryzias latipes; pma, Petromyzon marinus; ptr, Pan troglodytes; rno, Rattus norvegicus. (C) Predicted secondary structures of wt and mut (n.37C>T) miR-204 precursors obtained using the m-fold algorithm. The nucleotide mut in the family is indicated in blue and underlined. The 5p arm is shown in red, whereas the 3p arm is shown in green. Predicted sites for Drosha (black arrows) and Dicer (blue arrows) cleavage are indicated.
individuals (patients II-1, III-1, III-2, V-1, V-2, and V-3), which provided eight informative meioses. These data achieved a maximal LOD (logarithm of odds) score of 1.81, which falls below the level of 3.0 required to confirm significance of linkage. The data were therefore analyzed on the basis of linkage exclusion for those regions with a LOD score less than −2. There were four regions remaining over 5 Mb, which had a LOD score of between −2 and 1.81 (Fig. S1). Subsequently, exome sequencing was performed on patients II-1 and V-1 to identify possible causal variants within the potential linked regions. This revealed 39 variant calls common to both patients. To prioritize the likely disease causing mutations, we assumed a dominant model of inheritance and excluded homozygous changes. All variants reported in the Exome Variant Server of the National Heart, Lung and Blood Institute Exome Sequencing Project (evs.gs.washington.edu/EVS), dbSNP136, and the 1000 Genomes Project (www.1000genomes.org) were excluded. Variants that had been previously seen within an in-house database of 244 exomes and those variants confirmed by Sanger sequencing not to cosegregate with disease in the family were also excluded. This resulted in a single candidate variant. The n.37C > T mutation identified in the miRNA MIR204 (reference sequence NR_029621) was an excellent candidate for further study. MiR-204 is preferentially expressed in the eye, and several studies have determined its role in eye development (3, 9–11). Evidence of the critical function of miR-204 in early development strongly suggests a pathogenic role for the mutation identified in this family.

Sanger sequencing confirmed the MI R204 n.37C > T mutation segregates with the retinal dystrophy and coloboma phenotype in the family members who were tested (II-1, I-3, II-1, II-3, IV-1, and V-1) and were absent in the nonaffected individuals tested (V-2, V-3) (Fig. 2A). The mutation is positioned at the fourth nucleotide within the highly conserved seven-nucleotide seed region of the 5′ arm of the precursor miRNA [annotated according to mirBase, mirbase.org (12)] (Fig. 2B). Coinheritance of retinal dystrophy and MAC is considerably rare. Mutation analysis of an additional 21 cases of known retinal dystrophy with MAC with no family history identified no variants in miR-204. Screening of an additional cohort of 457 patients with isolated MAC and 672 patients with autosomal dominant retinal degeneration phenotypes revealed no novel mutations in miR-204. Moreover, this variant was not present in the control databases of the Exome Variant Server or 1000 Genomes Project. The Effect of the n.37C > T Mutation on miRNA Target Recognition. As the MIR204 mutation identified in our family lies within the seed region of the 5′ arm, and therefore in the stem region of premiR-204, we used the m-fold algorithm (mfold.rit.edu) (13) to assess how the mutation identified might alter the predicted RNA secondary structure of this microRNA. Although the n.37C > T mutation introduced a base-pairing mismatch, the decrease in free energy value was small, no bulges were introduced into the RNA structure, and the regions critical for Drosha and Dicer processing of pri-miRNA were not affected (Fig. 2C; nucleotides 32 and 92 for Drosha and nucleotides 54 and 71 for Dicer) (14). Therefore, the n.37C > T mutation was not predicted to destabilize the secondary structure of premiR-204 (Fig. 2C). Because mutations in miRNA sequence have been previously suggested to alter premiRNA processing (15), we examined the effect of the variant on the biogenesis of miR-204. Expression vectors containing the wild-type (wt) or the mutated (mut) form of precursor sequences of hsa-miR-204 were transiently transfected into HeLa cells, and expression levels of mature processed miRNA (miR-204) and of the precursor species (premiR) for both the wt and mut forms were assessed by quantitative RT-PCR (qRT-PCR), using specific custom-designed assays. Notably, the expression levels of both the miR-204 and premiR-204 species appeared largely unaffected by the n.37C > T mutation (17% decrease and 1.6% increase, respectively) compared with wt (Fig. S2A). This would suggest that these mechanisms do not account for the pathogenesis of this dominantly inherited phenotype.

Because the mutation lies within the seed region of the mature miRNA, it was more likely that this change would affect recognition of target genes. This could occur through two different modalities; that is, either impaired recognition of bona fide miR-204 targets (loss-of-function mechanism) or creation of novel aberrant target sites (gain-of-function mechanism). To evaluate the relative contribution of either of these mechanisms, we carried out a microRNA target prediction with the TargetScan (www.targetscan.org) and Diana-microT (diana.cslab.ece.ntua.gr/microT) tools, using as query the miR-204 sequence containing the n.37C > T variant. Intriguingly, we found that the mutated (mut)-miR-204 sequence was predicted to target a much higher number of mRNAs (mut-miR-204 targets; n = 1,129) compared with the wt sequence (wt-miR-204 targets; n = 557) (Dataset S1). Only 135 mRNA were predicted to be targeted by both the wt and the mut-miR-204 sequence, suggesting the n.37C > T sequence variant could indeed cause a notable alteration of miR-204 targeting properties.

To validate the above predictions and to obtain a more global assessment of the effect of the n.37C > T sequence variant in the miR-204 sequence, we carried out transcriptome analysis by RNAseq in a human RPE cell line (ARPE-19) (16) in which miR-204 is expressed at low levels (17). After transfecting ARPE-19 cells with microRNA mimics corresponding to either the wt- or mut-miR-204 sequence, we compared the results obtained from their RNAseq transcriptome profiles. We reasoned that if the target predictions were reliable, predicted wt-miR-204 targets should be enriched within the genes down-regulated in wt-transfected cells, whereas predicted mut-miR-204 targets should be enriched within the genes down-regulated in mut-transfected cells.

First, we found that the number of significantly down-regulated genes (fold change decrease of at least 10% at a false discovery rate < 0.05 with respect to a negative mimic control) was higher in mut-miR-204-transfected (n = 1,764) than in wt-miR-204-transfected (n = 1,200) ARPE-19 cells, supporting the notion that the mutation has a significant effect at the transcriptome level. We then looked at the representation of predicted targets within down-regulated genes and found that the predicted targets specific for wt-miR-204 were more significantly enriched in the genes down-regulated in cells transfected with wt mimics (150/1,200) (Fig. 3 A and C) than in genes down-regulated in cells transfected with mut mimics (84/1,764) (Fig. 3 B and C); P < 0.0001 (Fig. 3C). In contrast, predicted target genes specific for mut-miR-204 were more significantly enriched in the genes down-regulated in cells transfected with mut-mimics (249/1,764) (Fig. 3 E and F) than in the genes down-regulated in cells transfected with wt-mimics (107/1,200) (Fig. 3 D and F); P < 0.0001 (Fig. 3F). The complete lists of the wt-miR-204 and mut-miR-204 predicted targets found to be down-regulated are available, respectively, as Datasets S2 and S3.

To provide independent validation of the presented RNAseq analysis, we carried out qRT-PCR assays on two small subsets, respectively, of “newly generated” predicted targets, that is, those specifically down-regulated in mut-miR-204-transfected cells, but not in wt-miR-204–transfected cells, and of “lost” predicted targets, that is, those specifically down-regulated in wt-miR-204–transfected but not in mut-miR-204 transfected cells. The qPCR assays indeed validated the RNAseq results for 6/8 newly generated predicted targets and for 6/8 of the predicted lost targets analyzed (Fig. S2 B and C). Together, these data strongly suggested that the n.37C > T mutation leads to a significant alteration of miR-204 function, both by generating a
Fig. 3. The n.37C > T sequence variation confers altered targeting capabilities to miR-204, as assessed by transcriptome analysis. (A–F) Schematic representations of the behavior of predicted targets for either wt-miR-204 (A–C) or mut-miR-204 (D–F) in RNA-Seq experiments carried out in ARPE-19 cells transfected with either wt-miR-204 (A and D) or mut-miR-204 mimics (B and E). (A, B, D, and E) X axis, expression fold changes (on a log2 scale) versus control ARPE-19 cells (i.e., negative mimic transfected). Y axis, statistical significance of expression changes represented as false discovery rate values on a log10 scale. The black horizontal line indicates a false discovery rate of 0.05, and the black vertical lines indicate the 10% expression fold change thresholds chosen to identify genes showing significant expression changes. The blue vertical lines mark the 50% expression fold change value. Red dots indicate the genes that show statistically significant up-regulation in each experiment. Green dots indicate the genes that show statistically significant down-regulation in each experiment. Gray dots represent genes that do not display significant expression changes. Black dots in A and B represent genes that are predicted to be targets of the wt-miR-204, whereas in D and E, they represent genes that are predicted to be targets of the mut-miR-204, that is, carrying the n.37C > T variation. Mut-miR-204-specific predicted targets are significantly more enriched in the genes down-regulated in cells transfected with wt mimics (A) than in genes down-regulated in cells transfected with mut mimics (B), as quantified in the graph shown in C (P < 0.0001). Conversely, mut-miR-204-specific predicted targets are significantly more enriched in the genes down-regulated (DR) in cells transfected with mut-mimics (E) than in the genes down-regulated in cells transfected with wt-mimics (D), as quantified in the graph shown in F (P < 0.0001).
MiR-204 has a Physiological Role in Photoreceptor Function. MiR-204 has previously been shown to be expressed in the developing eye of medaka fish and mouse (3, 10), and we previously reported that knockdown of miR-204 in medaka fish was linked to microphthalmia, abnormal lens development, eye coloboma, and defects in axon projections of retinal ganglion cells (9, 10, 24). To confirm a physiological role of miR-204 also in the human eye, we performed RNA ISH experiments on adult human retina. This confirmed that miR-204 is expressed in the adult human retina and revealed expression in the retinal ganglion cell layer, inner nuclear layer, and retinal pigment epithelium. Importantly, this also demonstrated expression in the human photoreceptor cell layer (Fig. S5).

To test the possible contribution of miR-204 in photoreceptor homeostasis, we therefore interfered with miR-204 processing and activity in medaka fish, using a multiblocking morpholino (Mo), as previously described (9, 25), and assessed the consequences in the differentiated retina. Comparison of the morphology of the differentiated retina of control and Mo-miR-204–injected embryos revealed that morphant retinas displayed significant alteration in photoreceptor marker staining, which was more evident in ventral regions (Fig. S6). In particular, we observed by immunofluorescence a moderate reduction of rhodopsin-positive mature rod photoreceptors and of Zpr-1-positive mature cone photoreceptors (Fig. S6). In contrast, we did not observe in morphant fish any apparent alterations in the number of other retinal neuronal cells, as determined by expression analysis of Syntaxin, OS6, and oltcn2 (Fig. S6). We also observed an increased apoptosis, as determined by TUNEL assays, and no alterations in cell proliferation by phosphorylated histone H3 staining (Fig. S6). Finally, we recorded flash electroretinogram (ERG) under scotopic conditions from both control and miR-204 morphant larvae. We observed a significant reduction of the amplitude of the b-wave in miR-204 morphant compared with control medaka larvae (n = 30) (Fig. S6). These data confirm that miR-204 is necessary for normal photoreceptor differentiation and function in vertebrates.

Discussion

In this study we describe a five-generation family with an overlapping phenotype of inherited retinal dystrophy and optic fissure

Fig. 4. The n.37C > T mutation has a deleterious effect in vivo. (A–C) Bright-field microscopy images of lateral views eyes from control-injected (A), mut-miR-204–injected (B), and wt-miR-204–injected (C) embryos. Representative frontal eye sections immunostained with an anti-Rhodopsin (D–F; green) and Zpr-1 (G–I; green) antibodies, from st38 control-injected (D and G), mut-miR-204–injected (E and H), and wt-miR-204–injected (F and I) medaka fish. Sections are counterstained with DAPI (blue). We observed a significant reduction in the staining of both photoreceptor rods and cones in mut-miR-204–injected medaka fish compared with control-injected and wt-miR-204–injected animals. (J–L) Frontal vibratome sections of TUNEL-stained control-injected (J), mut-miR-204–injected (K), and wt-miR-204–injected embryos. A significant increase of cell death in the eyes of mut-miR-204–injected embryos was observed compared with both wt-miR-204– and control-injected embryos. Some TUNEL-positive cells are marked by blue arrows. (M) Quantification of TUNEL-positive cells in the retina of control-injected (blue bars), wt-miR-204–injected (red bars), and mut-miR-204–injected (green bars) embryos at three different embryonic stages; *P < 0.001 in likelihood ratio tests. L, lens; OT, optic vesicle; RPE, retinal pigment epithelium. (Scale bars, 100 and 20 μm.)

in retinal tissue (but not in the RPE) in mut-miR-204–injected embryos in comparison with both wt-miR-204– and control-injected embryos (Fig. 4 J–L). Notably, no significant differences were observed in the number of TUNEL-positive cells between wt-miR-204–injected and control retinas (Fig. 4 J and L). In contrast, we did not observe any alteration in the number of proliferating retinal cells in mut-miR-204–injected and control embryos (Fig. 4 J and L). MiR-204 encodes a transcription factor involved in the alteration, unlike that pre-

Overall, these data strongly support in vivo the pathogenic role of the n.37C > T variation in miR-204 in the complex phenotype observed in our family, which is likely exerted through a gain-of-function mechanism.

visible phenotype of wt-miR-204 overexpression was associated with a significant decrease in the number of phosphorylated histone H3-positive cells (Fig. S3). These findings indicate that the ocular phenotype observed in mut-miR-204–injected embryos is likely a result of activation of cell apoptosis.

Finally, we decided to analyze in vivo the behavior of two of the miR-204–specific predicted targets we had also validated by qRT-PCR assays in ARPE-19 cells; namely, Lef1 (lymphoid enhancer-binding factor-1) and Stk39 (serine threonine kinase 39) (Fig. S4). Lef1 encodes a transcription factor involved in the Wnt signaling pathway (22), whereas Stk39 encodes a serine/threonine kinase with a possible role in the cellular stress response pathway (23). Specifically, we analyzed by RNA ISH their expression in control, wt-miR-204–injected, and mut-miR-204–injected medaka embryos. Notably, we observed a reduction of their expression domains in mut-miR-204–injected embryos compared with both wt-miR-204 and control embryos (Fig. S4).

Conversely, no apparent alterations of their expression domains were observed in wt-miR-204–injected compared with control embryos (Fig. S4). Of note, we did not observe in mut-miR-204–injected embryos any gross lens alteration, unlike that previously described in miR-204 knockdown in medaka (10).

Overall, these data strongly support in vivo the pathogenic role of the n.37C > T variant in miR-204 in the complex phenotype observed in our family, which is likely exerted through a gain-of-function mechanism.

Discussion

In this study we describe a five-generation family with an overlapping phenotype of inherited retinal dystrophy and optic fissure
closure defect, iris coloboma. To elucidate the underlying molecular basis of this phenotype, we undertook a combined linkage and exome sequencing approach identifying four potentially linked regions, one of which, on chromosome 9q21, contained a previously undescribed heterozygous mutation in MIR204 (n.37C > T) that segregated with the phenotype observed in this family. Given the well-documented role of miR-204 in eye development, this mutation was a strong candidate for further investigation.  

MiR-204 has discrete and dynamic expression domains in the eye throughout development, being expressed in the RPE, neural retina, lens, and ciliary body in fish, mouse, and human (3, 4, 10, 11, 26–28). This miRNA has a closely related paralog in mammals; namely, miR-211, with which it shares the same seed sequence and very likely a similar set of targets and tissue expression pattern, although, in the latter respect, precise information is still lacking. A large body of evidence indicates that miR-204 plays a crucial role in the differentiation and function of all of the ocular structures in which it is expressed. In vitro assays have shown that miR-204 may be involved in the correct differentiation and function of RPE cells (27, 29, 30). MiR-204 is also required for proper lens development (9, 10). We previously reported that this action is exerted through a complex cross-talk with the transcription factor Pax6, a “master regulator” of eye development. We found that although Pax6 controls the expression of miR-204 (11), miR-204 can itself repress Pax6 through the targeting of the transcription factor Meis2 (10). Additional target genes that play a role in miR-204 action in lens differentiation are Ankrd13a (9), Sox11 (11), and Hmga1 (31). Finally, miR-204 controls the establishment of the proper dorsal-ventral axis, and its inactivation was linked to coloboma in medaka fish (10).  

Despite its pervasive role in many aspects of ocular differentiation, miR-204 has not previously been reported to be significantly expressed in photoreceptor cells (3, 11, 25, 26, 28) or to have an effect on photoreceptor function. Here, we provide for the first time to our knowledge RNA ISH data for miR-204 in the human retina, which also reveals its expression in photoreceptors (Fig. S5). We demonstrate that knockdown of miR-204 function in medaka fish leads to progressive alteration and death of photoreceptor cells (Fig. S6). These data strongly indicate that miR-204 is also required for photoreceptor development and function. The significant expression of this miRNA in the photoreceptor cell layer in human (Fig. S5) supports the hypothesis that miR-204 could exert its role on photoreceptor function through a cell autonomous mechanism. However, a contribution to a noncell autonomous action mediated by the strong expression of miR-204 in the RPE is also likely (3, 11, 28). There are many examples of RPE-specific genes that play essential roles in photoreceptor function and whose mutations have a pathogenic role in inherited retinal dystrophies (32, 33). The dissection of the molecular mechanisms through which miR-204 regulates photoreceptor function requires further study.  

This is the first report to our knowledge of a pathogenic mutation within miR-204 identified as the cause of an ocular disease. It is supported by two main considerations: First, the n.37C > T mutation falls within the mature miR-204 seed region, which is considered to be the most essential sequence domain for target recognition and down-regulation (34). We demonstrate in vitro that this variation has an effect on target miRNA recognition (Fig. 3 and Fig. S2), which would be anticipated to lead to significant dysregulation of gene expression in eye tissues both via natural or aberrantly generated miR-204 targets. Second, we demonstrate that the n.37C > T variation has a deleterious effect in vivo. In particular, we found that the overexpression of a miR-204 mimic carrying the n.37C > T mutation in medaka fish generates a dramatic eye phenotype characterized by gross malformations (including microphthalmia and coloboma) and photoreceptor abnormalities (Fig. 4). These results not only clearly support the deleterious role played by the n.37C > T mutation but also support its pathogenic role in the complex form of retinal dystrophy observed in the family we report in this study.  

We have identified a miR-204 mutation in a single large family. We did not identify this mutation, or additional miR-204 mutations, in patients with either isolated inherited retinal dystrophies or MAC that is, phenotypes partly overlapping that observed in our family. However, the combination of retinal dystrophy and ocular coloboma is very rarely observed in clinical practice, in particular when inherited as an autosomal dominant trait. It is therefore conceivable that this particular miR-204 mutation underlies the pathogenesis of an extremely rare ocular condition. This is in line with the limited number of reports of mutations within microRNAs associated with Mendelian disorders to date. The first report of mutations within a miRNA contributing to a disease phenotype was by Mencia et al., who identified two point mutations within the seed region of miR-96, which segregated with the affected patients from two separate Spanish families with autosomal dominant progressive hearing loss (35). The resulting mutations directly affected the miR-96 biogenesis and resulted in a significant reduction in the silencing of target genes. Soldà et al. reported a mutation in the 5p arm of MIR96 in a family with nonsyndromic inherited hearing loss (15). This mutation was found to impair correct maturation of miR-96, leading to a significant decrease in the expression level of the mature 3p and 5p microRNA arms. The only other example of a microRNA with point mutations being associated with a Mendelian disorder is represented by MIR184. Hughes et al. identified a mutation in the central nucleotide of the seed region of MIR184 as the cause of keratoconus and early-onset anterior polar cataracts in a large Irish family, although the underlying pathogenic mechanism was not extensively delineated (36). Iliff et al. reported the same mutation as the cause of EDICT syndrome, an autosomal dominant anterior segment dysgenesis syndrome characterized by endothelial dystrophy, iris hypoplasia, congenital cataract and stromal thinning (37). A further two novel heterozygous substitution variants, neither of which were located within the seed region, were later identified in MIR184 in two patients with isolated keratoconus (38).  

There are several reasons for the paucity of genetic diseases caused by miRNA mutations. First, given their shortness, it is expected that the number of potential mutations affecting miRNA mature sequences will be significantly lower compared with the number of mutations falling within functional sequence elements of protein coding genes. Second, given the fact that each miRNA can target hundreds of different genes, mutations affecting critical nucleotides of mature sequences may give rise to phenotypes that depend on the target specificity affected by each mutation. Mutations altering miRNA function may not only be localized within their mature sequences but also in their binding sites within the 3′-UTR of target genes, as described in the cases of spastic paraplegia (39) and X-linked chondrodysplasia (40). Such mutations in the 3′-UTR would be expected to selectively affect specific aspects of miRNA actions, and consequently to give rise to disease phenotypes that will differ from those observed when mutations lie in the miRNA sequence itself. The assessment of the true effect of 3′-UTR mutations on genetic disorders will require analysis of gene regions thus far neglected in mutation screens.  

The n.37C > T mutation results in an autosomal dominant phenotype and thus could, in principle, act either through loss of function (i.e., by impairing recognition of wt-miR-204 targets) or gain of function (by creating novel aberrant recognition sites in genes that should not normally be targeted by miR-204) mechanisms. We hypothesize that the gain-of-function mechanism exerts a relatively greater role for the following three reasons:
First, both bioinformatic predictions and transcriptome studies indicate that the n.37C>T mutation is able to generate a much larger dataset of predicted targets compared with the wt-miR-204 mature sequence (Fig. 3 and Dataset S1). Some of these were also validated both in vitro and in vivo (Figs. S2 and S4), including among the latter, Leftf. Leftf has previously been shown to play a role in retina (41–43) and RPE differentiation (44). Second, the injection of the mutant-miR-204 in medaka fish leads to a striking eye phenotype that is much more severe than that obtained with the wt-miR-204. In this respect, it is important to underline that the notable difference in phenotypic severity between the mutant-miR-204–injected medaka and the patients carrying the n.37C>T mutation will be contributed to by the fact that although in medaka there is ectopic overexpression of the mutant miRNA throughout the developing embryo, in patients the mutation exerts its deleterious effect only in the tissues and in the stages in which miR-204 is normally expressed.

Finally, there are previous clinical data that seem to further exclude haploinsufficiency. miR-204 is located within intron 8 of the TRP channel gene, TRPM3, on chromosome 9q21.12. Deletions within 9q21 and those specifically encompassing the TRPM3 gene have not been reported to cause ocular phenotypes, instead causing features of mental retardation, epilepsy, speech delay, autistic behavior, and moderate facial dysmorphism (18–21). Deletion of this region has been described in association with an autism phenotype in a family in which a paternal deletion of exons 1–9 of TRPM3, which included MIR204, was shared by two affected sons and an unaffected daughter who were not described to have an ocular phenotype or visual symptoms. Furthermore, at least 30 patients with deletions in this region are listed on the Decipher database, a web-based resource and database of genomic copy number variation data from analysis of patient DNA; none is described with coloboma or retinal disease, although one had optic nerve hypoplasia. Altogether, the above data indicate that the n.37C>T mutation mostly acts with a gain-of-function mechanism, although we cannot exclude that the loss of some miR-204 wt targets may still contribute to the phenotype observed in our family.

This study represents the first example to our knowledge of a microRNA-caused genetic disease most likely occurring via a gain-of-function mechanism; further studies will need to be undertaken to examine the possibility that this mechanism applies to other microRNA-caused genetic disease. This is also the first example to our knowledge of a microRNA mutation with a causative role in inherited retinal dystrophies in patients, as to date, microRNAs have been linked to such diseases only in mouse (45).

In summary, we have identified a new genetic cause for the pathogenesis of complex forms of eye diseases particularly characterized by the combination of retinal dystrophy and developmental abnormalities. Our findings contribute to highlighting miR-204 as a new putative ‘master regulator’ of eye development and, in particular, has been instrumental in uncovering its newly identified role in photoreceptor survival and function. Finally, our results shed further light on the recognition of the role of miRNAs as primary pathogenic agents in human genetic diseases.

Materials and Methods

Subjects. A single five-generation family with a highly unusual and visually disabling phenotype consisting of retinal dystrophy and bilateral iris coloboma presented at the retinal and genetic clinic of the Manchester Royal Eye Hospital in the United Kingdom. A total of nine family members exhibited a similar phenotype (six female and three male) (Fig. 1A). To elucidate the molecular basis of this phenotype, we enrolled eight family members for clinical and molecular genetic analysis, including six affected individuals. Ethical approval and informed consent were obtained from all study participants. The research adhered to the tenets of the Declaration of Helsinki.

Disease status was determined by a full medical, ophthalmic, and family history combined with clinical examination and electrophysiological evidence in proband V-I and his mother, patient IV-1. Medical records for other family members were reviewed with the patients’ written consent. Ophthalmic examination included anterior segment examination by slit lamp biomicroscopy with evaluation of anterior chamber depth using the Von Herrick method, intraocular pressure measurements, and dilated fundus examination and photography. Optical coherence tomography (OCT) for retinal thickness assessment using the Heidelberg spectral-domain OCT (Heidelberg engineering) was performed with autofluorescence and color imaging. Full-field flash ERGs were also carried out in individual V-1. Individual IV-1 underwent ERG testing in the 1980s. ERGs were recorded following the standards of the International Society for Clinical Electrophysiology of Vision (46). Both scotopic rod-driven responses and cone-driven photopic single flash and 30-Hz flicker stimuli were recorded sequentially.

Linkage Analysis. Genomewide SNP analysis was carried out using the Affymetrix Genome-Wide SNP6.0 microarray. Genotypes were generated using the genotyping console software provided by Affymetrix, using the Birdseed V2 algorithm and a confidence threshold of 0.005. A subset of SNPs was selected for linkage analysis based on having a minor allele frequency of 0.2 to reduce the problems associated with linkage disequilibrium when using smaller data sets. Parametric multipoint linkage analysis was performed using Merlin (csg.sph.umich.edu/binacula/merlin/index.html), using a dominant model with full penetrance.

Exome Sequencing and Variant Prioritization. Whole-exome target enrichment and sequencing were performed on 3 µg of DNA extracted from peripheral blood of two affected family members, proband V-I and patient II-I (Fig. 1A). For patient V-I, enrichment was performed using the SureSelect Human All Exon v1 Enrichment kit (Agilent Technologies) for the Illumina HiSeq system, according to the manufacturer’s protocol. Sequencing was carried out on a HiSeq, 2000 sequencer (Illumina Inc.), following the manufacturer’s protocols (Hong Kong Co.). Sequence data were mapped to the hg19 reference human genome, using BWA software. Variants were called using GATK v2.4.7 software and then filtered for those SNPs with ≤5x coverage. Approximately 4.5 GB of sequence was mapped to the genome reference hg19, with 69.4% of the exome covered at 20-fold or higher. SNPs and insertions/deletions were initially annotated to genes using Ensembl v68, and the functional consequences were then defined against an in-house list of Refseq transcripts (www.ncbi.nlm.nih.gov/refseq). Using Ensembl’s defined consequence hierarchically system, the highest affecting consequence for a variant in a gene was retained. To find likely pathogenic changes, an in-house hierarchy system of functional consequence was used to prioritize variants, as detailed in ref. 47. Briefly, variants were filtered out if they were present in dbSNP136 (unless seen in the Human Gene Mutation Database, www.hgmd.org), seen more than once in our in-house variant database, or present in the 1000 Genomes Project or the Exome Variant Server. Novel changes were analyzed further using the in-silico tools SIFT (sorting intolerant from tolerant) (48), PolyPhen-2 (49), and splicing prediction tools via the software Alamut (Interactive Biosoftware, LLC) to assess their pathogenicity.

For patient II-1, enrichment was performed using TargetSeq exome enrichment (Life Technologies), following the manufacturer’s protocols. Emulsion PCR was conducted on the resulting enriched sample library, and the sample was run indexed with unrelated samples on a SOLID 5500x1 sequencer following the manufacturer’s protocols. Sequence data were mapped to the hg19 reference human genome, using Lifescope software. Approximately 4.5 GB of sequence mapped uniquely to the genome reference hg19, with 68.2% of the exome covered at 20-fold or higher. Variants were called using a combination of the Lifescope software suite and SAMtools (freevare) (50) and then filtered for those SNPs with ≤5x coverage. SNPs were annotated and filtered as for patient V-I.

PCR and Sanger Sequencing to Confirm Novel Variants. Mutations identified in candidate genes by exome sequencing were confirmed by direct Sanger sequencing. PCR amplification of MIR204 (M10000284) was performed using the following oligonucleotide primers: F-5′-GGACTCTTCTGATGCGTGTA-3′ and R-5′-TTTACCTCTTCTAAAAACTCAAGA-3′. Amplicons were sequenced using the BigDye Terminator v3.1 system (Life Technologies) and analyzed on an ABI 3730 DNA Analyzer (Life Technologies).

Cell Transfections, RNA Extraction, and Analysis. ARPE-19 cells (16) were cultured at 37 °C in a humidified chamber supplemented with 5% (vol/vol) CO2. Cells were seeded at 135,000 cells/mL in 24-multwell plates and transfection...
ERGs were recorded as described previously (55). Briefly, medaka fish (Oryzias latipes) were used (25). Me- dikas were dark adapted for 30 min. For recording, a reference electrode was placed in a 1% agarose in ddH₂O. The larva was placed dorsal-up on a moist paper covering the reference electrode. The recording electrode with a tip diameter of ~20 μm was filled with buffer E3 (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, and 0.33 mM MgSO₄) and placed on the cornea of the larva. <hypertonic saline was applied to regulate the osmotic conditions.>Light stimuli of 100 ms with intermittent intervals of 7 s were applied. The light stimulus intensity was 663 lx.

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qRT-PCR to Assess Biogenesis of miR-204. psulx plasmid constructs containing the wt or the mutant form of precursor sequences of hsa-miR-204, generated as previously described (9, 10), were transfected into HeLa cells using Lipofectamine 2000 (Life Technologies), according to the manufacturer’s instructions. RNA was extracted after 48 h, using the mirNeasy isolation kit (Qiagen). Reverse transcription was performed using the Taqman MicroRNA reverse transcription kit (Life Technologies) with specific custom-designed stem loop RT primers for the mature miR-204 (5′-GGGCGCTTGGTCTATGCT-3′) and pre-miR-204 (5′-GGTCTGATGCAGAGGCTGGTATCTGAGGGTCCGAGGTATTCGCACTGGATACGACAGGCAT-3′) products. Real-time PCR was performed using FastStart Universal SYBR Green Master mix (Roche Applied Sciences) and miRNA-specific forward and reverse primers (mature miR-204: F-5′-CGGCGCTTGGTCTATGCT-3′ and R-5′-GGGCGCTTGGTATCTGAGGGTCCGAGGTATTCGCACTGGATACGACAGGCAT-3′). Expression values were generated using a standard curve method. In all experiments, EGFP was cotransfected as an internal control to normalize for transfection efficiency, and the expression was assessed using a Taqman gene expression assay (Life Technologies) according to the manufacturer’s instructions. Each assay was performed in triplicate, and data are presented as the mean ± SD of three individual experiments.

Medaka Stocks and miR-204 Mimo Injections. The Cab-strain of wild medaka fish (Oryzias latipes) was maintained as previously described (9). Embryos were staged according to Iwamatsu et al. (53). MoS (Gene Tools, LLC) and both wt and mut miRDIAN Dharnaco miR-204 mimics were designed and injected into one-cell fertilized embryos, as previously described (10). The miR-204 Mo used in this study targets both identical copies of the mature miR-204 present in the medaka genome (10). All fish studies were conducted in strict accordance with the institutional guidelines for animal research and approved by the Italian Ministry of Health.

RNA in Situ Hybridization and Immunofluorescence. Human retinal sections were obtained from cornea donors collected by the Fondazione Banca degli occhi del Veneto (in compliance with the tenets of the Declaration of Helsinki). For miR-204 detection, we used the miRCURY LNA microRNA ISH Optimization kit at 53 °C, according to the manufacturer’s instructions. In situ hybridization of 30 nM of probe was performed using the miRCURY LNA microRNA ISH Optimization kit at 53 °C, according to the manufacturer’s protocol (Exiqon), with minor modifications (3). As a negative control, a double-DIG-labeled LNA scrambled microRNA probe, included in the above kit, was hybridized in parallel at the same concentration and optimal hybridization temperature. Whole-mount RNA ISHs were performed, photographed, and sectioned (25-μm vibratome sections), as described by Conte et al. (25). Digoxigenin-labeled anti-sense and sense riboprobes for olCx1, olPax6, olOtx2, and olRhodopsin were used (25). Medaka embryos were cryo-sectioned (12-μm) and immun-labeling was performed as previously described (25), using mouse monoclonal antibodies to Rhodopsin (1:2,000; Sigma) and Zpr1 (1:2,000; ZIRC) and the rabbit polyclonal antibody to Prox1 (1:200; Chemicon). Alexa-488-conjugated goat α-mouse and goat α-rabbit (1:1,000; Invitrogen) secondary antibodies were used. For the cell proliferation analysis, rabbit α-phospho-histone H3 (1:100; Cell Signaling Technology) and peroxidase-conjugated anti-rabbit antibody (1:200; Vector Laboratories) were used followed by 3,3′-diaminobenzidine staining, as previously described (54). Confocal images were acquired using the Zeiss microscope, LSM 710.

Detection of Apoptotic Cell Death. The extent and distribution of apoptotic cell death was determined by TUNEL, using the In Situ Cell Death Detection Kit, POD (Roche), following the manufacturer’s protocol.

Fish ERGs. ERGs were recorded as described previously (55). Briefly, medaka larvae were dark adapted for 30 min. For recording, a reference electrode was placed in a 1% agarose in ddH₂O. The larva was placed dorsal-up on a moist paper covering the reference electrode. The recording electrode with a tip diameter of ~20 μm was filled with buffer E3 (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, and 0.33 mM MgSO₄) and placed on the cornea of the larva. Light stimuli of 100 ms with intermittent intervals of 7 s were applied. The light stimulus intensity was 663 lx.


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