REVIEW

Photoreceptor cell fate specification in vertebrates

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ABSTRACT

Photoreceptors – the light-sensitive cells in the vertebrate retina – have been extremely well-characterized with regards to their biochemistry, cell biology and physiology. They therefore provide an excellent model for exploring the factors and mechanisms that drive neural progenitors into a differentiated cell fate in the nervous system. As a result, great progress in understanding the transcriptional network that controls photoreceptor specification and differentiation has been made over the last 20 years. This progress has also enabled the production of photoreceptors from pluripotent stem cells, thereby aiding the development of regenerative medical approaches to eye disease. In this Review, we outline the signaling and transcription factors that drive vertebrate photoreceptor development and discuss how these function together in gene regulatory networks to control photoreceptor cell fate specification.

KEY WORDS: Retina, Photoreceptor, Cell fate specification, Fate potential, Multipotent, Competence

Introduction

The retina is the part of the eye that senses light and communicates this information to the brain via the optic nerve. The vertebrate retina comprises several major cell types – retinal ganglion output neurons, bipolar, horizontal and amacrine interneurons, Müller glia and photoreceptors – each of which carries out specialized functions. The detection of light stimuli is mediated by photoreceptors, which are highly specialized neurons that occupy the outermost layer of the retina (Fig. 1A). Vertebrate photoreceptors have a unique polarized morphology (Fig. 1B) that includes an outer segment filled with photoreceptive pigments (opsins), a connecting cilium, the inner segment, the nucleus, the axon and the synaptic terminal (Rodieck, 1998; Swaroop et al., 2010). Normal photoreceptor function depends upon maintaining these compartments. There are two basic subtypes of photoreceptors: rods and cones. Rod photoreceptors are highly sensitive and function in dim light to mediate night vision; they use rhodopsin as the light-sensitive photopigment. Cone photoreceptors are present as several subtypes, each of which contains one or more opsins that are maximally sensitive to a different wavelength of light; cones thus mediate color perception.

Rods and cones, like other neurons in the central nervous system, express genes that are required for synapse formation and neurotransmitter release. The process of phototransduction requires the expression of many genes that uniquely mark photoreceptors. Historically, this distinction has facilitated the discovery of gene regulatory networks that control photoreceptor specification (see Glossary, Box 1) and physiology. Understanding the full cascade of gene regulatory events that control the

specification of photoreceptor fate (see Glossary, Box 1) remains a fascinating and unresolved problem in developmental biology. Diseases that affect the genesis, survival or function of photoreceptors are major causes of vision loss (Swaroop et al., 2010) (Box 2). With recent increases in the prospect of regenerative therapies to reverse vision loss, interest in uncovering the mechanisms that govern photoreceptor development has grown.

In this Review, we first provide an overview of retinal development. This topic has been discussed in many recent reviews (Agathocleous and Harris, 2009; Brzezinski and Reh, 2010; Bassett and Wallace, 2012; Xiang, 2013; Boije et al., 2014; Cepko, 2014) and is covered only briefly here. We then discuss the current model of photoreceptor development that has emerged from over 25 years of studies on retinogenesis. This model of photoreceptor fate specification has had practical applications in the field of regenerative medicine; some of the key signaling molecules identified from developmental studies are now being used to enhance photoreceptor genesis from pluripotent stem cells. Throughout the Review, we highlight gaps in our understanding of photoreceptor fate specification and we finish by discussing several important questions that remain unresolved.

Features of retinal and photoreceptor development

Photoreceptors are generated from a population of proliferative progenitors (see Glossary, Box 1) in the retinal neuroepithelium, a derivative of the neural tube. Rod and cone photoreceptors, along with all of the other retinal cell types, are generated from what are known as multipotent progenitors. Cell lineage-tracing studies in mice, frogs and fish have shown that these progenitor cells can divide both symmetrically and asymmetrically, and that their terminal division can generate different retinal cell types (Turner and Cepko, 1987; Wetts and Fraser, 1988; Turner et al., 1990). These lineage studies were widely interpreted to demonstrate that progenitors are not limited to producing a single type of retinal neuron, i.e. there are no progenitors that are solely restricted to producing photoreceptors. However, this is not always the case; it has been known for many years that teleost fish (e.g. zebrafish and goldfish) possess a progenitor that is restricted to producing only rod photoreceptors, although this has been considered an adaptation to maintain light sensitivity and rod density as the retina grows in postembryonic stages (Raymond and Rivlin, 1987), and more recent studies have provided evidence for horizontal- and cone-restricted progenitors in fish (Godinho et al., 2007; He et al., 2012; Suzuki et al., 2013). Together, these studies suggest that many of the specification events leading to the generation of photoreceptors, and even their specific subtype, can occur in either proliferative progenitor or postmitotic precursor (see Glossary, Box 1) cells.

Experiments that correlate the timing of progenitor cell cycle exit ('birth') with cell fate have shown that the different retinal cell types are formed in a partly overlapping sequence that is largely conserved among vertebrates (Sidman, 1961; Young, 1985; Altschuler et al., 1991; Rapaport et al., 2004; Wong and Rapaport, 2009). When integrated with lineage-tracing data, these

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A Retinal anatomy **B** Photoreceptor structure **C** Photoreceptor birthdating in mice
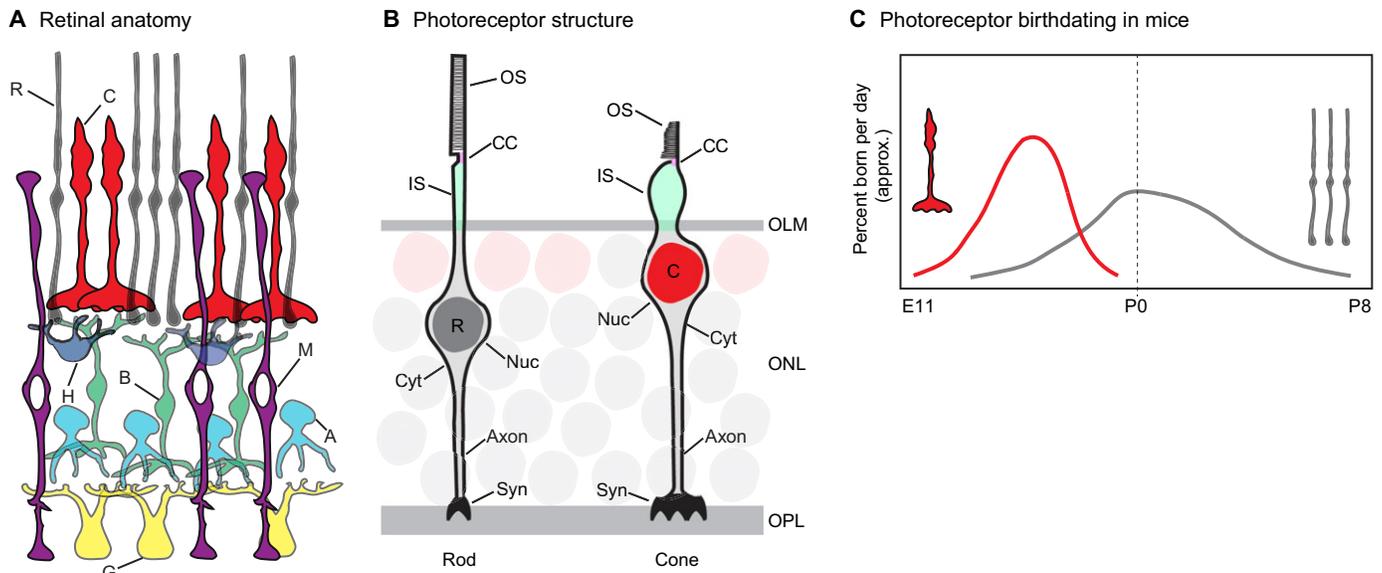
Fig. 1. Retinal anatomy and photoreceptor birthdating profiles. (A) The vertebrate retina comprises seven major cell types. Light-sensitive rod (R, gray) and cone (C, red) photoreceptors occupy the outermost layer of the retina, with rods outnumbering cones (35-to-1 in mice, 20-to-1 in humans). Photoreceptors synapse with bipolar (B, green) and horizontal (H, blue) interneurons. Signals from bipolar cells are relayed to retinal ganglion cells (G, yellow), the axons of which project to multiple areas of the brain. Amacrine (A, cyan) interneurons are located in the inner retina, and Müller glia (M, purple) cell bodies span the thickness of the retina. (B) Rod and cone photoreceptors exhibit highly polarized structures. Light is captured by opsins located in membrane discs (in rods) or folds (in cones) of the outer segment (OS), a modified primary cilium. The inner segment (IS, green) is a specialized area of cytoplasm (Cyt) starting at the outer limiting membrane (OLM) of the retina. It is a site of concentrated biosynthesis and the resulting products are transported through the narrow connecting cilium (CC, pink) into the OS. Cone inner segments tend to be larger than those of rods. Photoreceptor nuclei (Nuc) are densely packed within the outer nuclear layer (ONL), with cone nuclei (C, red) clustered nearer the OLM compared with rod nuclei (R). Photoreceptors span the ONL and extend their axons to the outer plexiform layer (OPL), where they form synapses (Syn) with horizontal and bipolar interneurons. Cones exhibit large multisynaptic pedicles, whereas rods have a smaller synaptic spherule. (C) Timing of permanent cell cycle exit ('birthdate') in the developing mouse retina. Neural birthdates follow a central-to-peripheral gradient, starting at embryonic day (E) 11 and finishing around postnatal day (P) 7. Cones (red) are born embryonically, with production peaking at E14.5 and E15.5 in the central and peripheral areas of the retina, respectively. The considerably more numerous rods (gray) are born at all but the earliest times in retinogenesis, peaking at P0 centrally and between P0 and P2 in the peripheral parts of the retina. The area under the curve represents 100% of the cells born for each type. See Carter-Dawson and LaVail (1979) for quantitative details.

birthdating studies indicate that the frequency of each cell type generated by progenitors changes over time. In line with this, the isolation of retinal progenitors from different stages of development further demonstrated that the probability of generating specific fates *in vitro* changes over the period of neurogenesis (Reh and Kljavin, 1989; Watanabe and Raff, 1990). These data led several investigators to propose a model in which progenitors change their potential (see Glossary, Box 1) to control fate specification (Reh and Cagan, 1994; Cepko et al., 1996; Frantz and McConnell, 1996). In these models, both the acquisition and the removal (restriction) of potential are critical specification events. The change in potential is likely to be due to a combination of intrinsic and extrinsic factors, such as transcription factors and signaling molecules, respectively. For example, progenitors from early stages of retinal development do not express transcription factors such as Sox9 and Ascl1 (Jasoni and Reh, 1996; Georgi and Reh, 2010; Brzezinski et al., 2011), and are unresponsive to epidermal growth factor (EGF), but those from later times express these transcription factors and respond to EGF (Anchan et al., 1991; Lillien, 1995). In addition to transcription factors and signaling molecules, cells may restrict their potential by changing their epigenetic landscape, such that fate-determining transcription factors no longer have access to chromatin at key target sequences. In the absence of either event, a progenitor could still be multipotent, but such a cell would only be able to produce a restricted range of cell fates at that particular time. Such dynamic fate potential models require a clock-like mechanism, which again

might be intrinsically controlled or rely on signals from surrounding cells. A discussion of the mechanisms that underlie this 'clock' of neurogenesis, and the epigenetic control of photoreceptor development (Yang et al., 2015), are beyond the scope of this Review, although it should be noted that some of the key transcription factors and miRNA networks (e.g. *Let7*, *Lin28*) that control analogous pathways in other systems have been shown to regulate developmental timing in the retina as well (La Torre et al., 2013).

Transcription factors important for the generation of photoreceptor precursors from multipotent progenitors

Photoreceptors are generated from retinal progenitors over most of the course of retinal development. In general, the first cone photoreceptors are formed before the initiation of rod photoreceptor production (Fig. 1C) (Carter-Dawson and LaVail, 1979). Within mammals, this is particularly evident in those species that exhibit an extended gestation, such as the monkey, where the first cone photoreceptors are born more than a week before the onset of rod photoreceptor production (La Vail et al., 1991). To better understand how retinal progenitors produce photoreceptors, a number of studies have focused on the transcription factors expressed by progenitors and early photoreceptors (see Table 1).

One of the key transcription factors necessary for photoreceptor development is Otx2. In the mouse retina, most proliferative progenitors do not express detectable levels of Otx2, but this transcription factor is rapidly upregulated as cells exit the mitotic

Box 1. Glossary
Our comments on photoreceptor development are framed in the larger context of cell fate specification. Much of our terminology is derived from this broader literature, but because some of these terms have been used in multiple ways we explicitly state the manner in which we are using them below.

Fate: A cellular state characterized by a unique combination of stable gene expression patterns and their downstream functional effects. Also referred to as 'cell type', 'identity' and 'terminal differentiation'.

Specification: The process by which a cell acquires its fate. This process can have several steps, or intermediates, before reaching the final stable gene expression state that defines a given cell fate.

Commitment: The point during specification when a cell becomes constrained to execute a specific gene expression profile (often repressing alternative fates). This event may greatly precede the full gene expression state that characterizes a cell fate.

Potential: The range of fates available to a cell. A multipotent cell has more than one gene expression state that can be stabilized. Potential can be considered on two time-scales: (1) instantaneously, i.e. those fates that a cell can adopt at a given point in development; and (2) integrated over time, i.e. those fates that the cell and any of its progeny can adopt throughout the development of the organism.

Progenitor: A proliferative cell that can give rise to one or more distinct cell fates.

Precursor: A non-proliferative (i.e. postmitotic) cell that does not yet express all the characteristics of a mature cell type. This includes cells that have not committed to a fate.

In the context of photoreceptor development, the process by which a multipotent cell achieves a specific fate via specification and commitment occurs in both progenitors and precursors. Cells progress through a series of partially stable gene expression states until a photoreceptor-specific state can be made permanent. The transcriptional networks that regulate these gene expression states are only partially understood, but experimental manipulations of transcription factors required for photoreceptor development reveal that the specification events can occur in both progenitors and precursors. As the precursor cells stabilize the mature pattern of photoreceptor gene expression they become more resistant to change. This later step of 'lock-down' in fate might be due to epigenetic regulation.

Box 2. Photoreceptor loss and dysfunction
Photoreceptors are highly metabolically active cells that require a complex polarized morphology and the presence of nearby support cells both to function normally and to survive. Because of these demanding features, photoreceptor health is especially sensitive to genetic and environmental perturbations. Several human diseases affect photoreceptors, either directly or by altering supporting cell types. These diseases can affect one or both photoreceptor types with widely differing onsets, rates and severities. Vision loss ranges from reduced visual fields or acuity (common) to the total absence of light perception (rare). Photoreceptor disease can be categorized loosely into congenital and acquired forms. Mutations in many different genes can disrupt photoreceptor development, morphology or physiology leading to congenital disease. These mutations are highly heterogeneous and cause a wide spectrum of vision loss in patients. Most mutations result in progressive late-onset diseases (e.g. retinitis pigmentosa), but some can cause severe early-onset diseases (e.g. Leber's congenital amaurosis). Similar vision loss can occur when genes required for the function of photoreceptor support cell types (e.g. retinal pigmented epithelial cells) are mutated. In addition, some mutations can cause syndromic diseases by affecting cells that share developmental pathways or key morphological features, such as the highly specialized primary cilium found in photoreceptors and a few other cell types (e.g. Senior-Løken and Bardet-Biedl syndromes). Acquired photoreceptor diseases are much more common than congenital disorders, affecting millions of people in the USA alone. These diseases (e.g. age-related macular degeneration and diabetic retinopathy) primarily affect supporting cell types and thus indirectly cause photoreceptor dysfunction and cell death. Whether congenital or acquired, photoreceptor cell death causes permanent vision loss because neither rods nor cones regenerate.

cycle (Muranishi et al., 2011). Otx2 appears to act early in the process by which photoreceptors and bipolar cells acquire their unique identities. The deletion of *Otx2* from the retina prevents the development of photoreceptors and bipolar cells, whereas its overexpression can promote the formation of both cell types (Nishida et al., 2003; Koike et al., 2007; Sato et al., 2007; Wang et al., 2014). Accordingly, chromatin immunoprecipitation (ChIP) and enhancer characterization experiments show that Otx2 associates with the promoters and enhancers of genes expressed in both photoreceptors and bipolar cells (Kim et al., 2008; Brzezinski et al., 2013; Samuel et al., 2014).

The initiation of Otx2 expression in progenitors leads to the activation of additional transcription factors required for correct fate specification. Two of these factors, Vsx2 (Chx10) and Prdm1 (Blimp1), act downstream of Otx2 and control whether Otx2-expressing cells develop as photoreceptors or bipolar cells (Fig. 2A). Vsx2 is expressed by progenitors and, after cell cycle exit, is upregulated in bipolar cells directly downstream of Otx2 (Kim et al., 2008). Overexpression and ChIP experiments have shown that Vsx2 represses photoreceptor-specific genes (Dorval et al., 2005, 2006; Livne-Bar et al., 2006). It was also shown that Vsx2 regulates progenitor cell proliferation, and that progenitors in *Vsx2* mutant mice fail to generate bipolar cells even when their proliferation defects are rescued (Burmeister et al., 1996; Green et al., 2003). Otx2 also directly activates Prdm1 (Brzezinski et al.,

2010; Katoh et al., 2010; Wang et al., 2014). In *Prdm1* mutants, cells begin to adopt photoreceptor identity but instead switch to a bipolar cell fate (Brzezinski et al., 2010, 2013; Katoh et al., 2010). Thus, early-born Otx2-expressing cells, which are normally limited to photoreceptor fates, can generate bipolar cells in these mutants, suggesting that Prdm1 prevents Otx2-expressing cells from adopting a bipolar fate. These data support a model in which Otx2 expression provides precursors with the potential to become photoreceptors and bipolar cells, and then the Otx2 targets Prdm1 and Vsx2 restrict precursors to either a photoreceptor or bipolar fate (Fig. 2A), respectively. How the balance between these fate choices is dynamically regulated over developmental time is unknown.

Another transcription factor that is important for eye development is Pax6, which is expressed in all progenitors. Retinas lacking *Pax6* fail to form photoreceptors and most other cell types (Marquardt et al., 2001). Interestingly, some photoreceptor genes (e.g. *Crx*) are inappropriately activated in these mutants (Oron-Karni et al., 2008). These data suggest that Pax6 is required in progenitors for photoreceptor (and other cell type) potential, but that it also temporarily restricts the ability of progenitors to activate a photoreceptor program.

Since most progenitors are not restricted to producing only a single cell type, such as a photoreceptor, what determines which precursor cells express Otx2 and acquire a photoreceptor fate? One possibility is that only a subset of the progenitor population has the ability to activate Otx2. This could be a stochastic process in progenitors or the result of a preprogrammed process of progenitor potential regulation. Another possibility is that the activation of Otx2 in progenitors is a default outcome, which must then be repressed to generate other cell types. In support of this latter hypothesis, experiments with dissociated chick retinal cell cultures suggest that all progenitors are able to express Otx2 (Adler and

Table 1. Transcription factors expressed in retinal progenitors and precursors

Transcription factor* (synonyms)	Type	Loss-of-function effects on mouse photoreceptor development[‡]	References
Ascl1 (Mash1)	bHLH	Reduction in late-born fates	Tomita et al., 1996; Brzezinski et al., 2011
Atoh7 (Math5)	bHLH	Modest cone excess	Brown et al., 2001
Crx	Homeodomain	Photoreceptors present but do not function	Chen et al., 1997; Freund et al., 1997; Furukawa et al., 1997
Foxn4	Winged helix-turn-helix	Excess photoreceptors	Li et al., 2004
Neurod1	bHLH	Loss of M-cones, excess S-opsin expression in cones	Morrow et al., 1999; Liu et al., 2008
Neurog2 (Ngn2)	bHLH	None	Hufnagel et al., 2010
Notch1	RAM	Excess photoreceptors[§]	Jadhav et al., 2006; Yaron et al., 2006
Nr2e3	Nuclear hormone receptor	Many photoreceptors co-express both rod and cone genes, excess S-opsin[+] cones, rod functional deficits	Akhmedov et al., 2000; Corbo and Cepko, 2005
Nrl	bZIP	Conversion of rods into S-opsin[+] cones	Mears et al., 2001
Olig2	bHLH	None	Hafler et al., 2012
Onecut1 (Oc1)	Cut domain, homeodomain	Fewer total cones, loss of M-cones, excess S-opsin expression in cones[¶]	Sapkota et al., 2014
Otx2	Homeodomain	No photoreceptors	Nishida et al., 2003; Sato et al., 2007
Pax6	Paired domain, homeodomain	No photoreceptors	Marquardt et al., 2001
Prdm1 (Blimp1)	Zinc finger	Excess bipolar cells at the expense of photoreceptors	Brzezinski et al., 2010; Katoh et al., 2010
Ptf1a	bHLH	None	Fujitani et al., 2006
Rorb	Nuclear hormone receptor	Loss of rods, increased cones, photoreceptors non-functional	Fu et al., 2014
Rxrg	Nuclear hormone receptor	Excess S-opsin expression in cones	Roberts et al., 2005
Thrb2 (Thrb)	Nuclear hormone receptor	Loss of M-cones, excess S-opsin expression in cones	Ng et al., 2001; Roberts et al., 2006
Vsx2 (Chx10)	Homeodomain	Increase in rods at the expense of bipolar cells[#]	Livne-Bar et al., 2006

bHLH, basic helix-loop-helix; bZIP, basic leucine zipper; RAM, Rbpjk (recombination signal binding protein for immunoglobulin kappa J region)-associated module.
*Mouse genes.
[‡]Effects on other cell types not listed.
[§]Early loss of Notch1 increased the number of cones formed, whereas later loss increased rod numbers.
[¶]When both Onecut1 and Onecut2 genes are simultaneously removed.
[#]Effects on cone photoreceptor formation are difficult to ascertain as Vsx2 is also required for normal progenitor proliferation.

Hatlee, 1989). Similarly, blocking Notch signaling in cultured mouse retinas can drive Otx2 expression and photoreceptor formation at a high rate (Nelson et al., 2007). Recently, lineage-tracing studies have revealed distinct subsets of proliferative progenitors that exhibit restricted fate potential and express varying combinations of transcription factors such as Ascl1 and Olig2. The fate mapping of *Ascl1*-expressing cells demonstrated that they give rise to all types except retinal ganglion cells, suggesting that Ascl1 marks a single fate-restricted lineage in the retina (Fig. 2B) (Brzezinski et al., 2011). Similarly, Olig2-expressing cells can give rise to all but ganglion cells and Müller glia, representing ~96% of the cells in the adult mouse retina (Fig. 2B) (Jeon et al., 1998; Hafler et al., 2012). Furthermore, some of these Olig2-expressing cells were poised between two outcomes; for example, between cone and horizontal cell fates, or between rod and amacrine fates. Similar to the *Olig2* lineage-tracing results, *Prdm1* fate-mapping experiments revealed that the Prdm1-expressing population can give rise to all cell types except ganglion cells and Müller glia (Brzezinski et al., 2013). Given that Prdm1 is made only by Otx2-expressing cells, these results imply that the Otx2-expressing cohort can generate photoreceptors, bipolar cells, amacrine cells and horizontal cells. This result is consistent with the hypothesis that the activation of Otx2 in progenitors is a default outcome, which must then be repressed to

generate other retinal cell types, although definitive Otx2 lineage-tracing studies are required to further test this hypothesis.

If the expression of Otx2 is indeed the default program, how then does the retina make cells other than bipolar cells and photoreceptors? Recent insights have come from characterizing other transcription factors expressed in retinal progenitors. For example, the transcription factors Foxn4 and Rorβ are expressed by progenitors (Chow et al., 1998; Li et al., 2004; Luo et al., 2012; Liu et al., 2013; Fu et al., 2014) and combine to induce the expression of *Ptf1a*, which encodes a transcription factor that is necessary for amacrine and horizontal cell genesis (Fujitani et al., 2006; Liu et al., 2013). Lineage tracing of *Ptf1a*-expressing cells labels only horizontal and amacrine cells (Fujitani et al., 2006), suggesting that any Otx2-expressing cells that also express *Ptf1a* have already (or will) become restricted to these two fates. The observation of two-cell clones containing a photoreceptor and an amacrine/horizontal outcome (Hafler et al., 2012) also suggests that this potential restriction occurs during the last division or in postmitotic precursors in mice. By contrast, Otx2-expressing precursors do not appear to generate ganglion cells, the first cell type generated in the retina. The ability to form these cells is mediated by *Atoh7* (*Math5*), but lineage-tracing studies show that only a small subset of cells acquires the potential to generate ganglion cells (Brown et al., 2001; Wang et al., 2001; Yang et al., 2003; Feng et al., 2010; Brzezinski

DEVELOPMENT

A Potential acquisition and restriction

B Retinal lineages
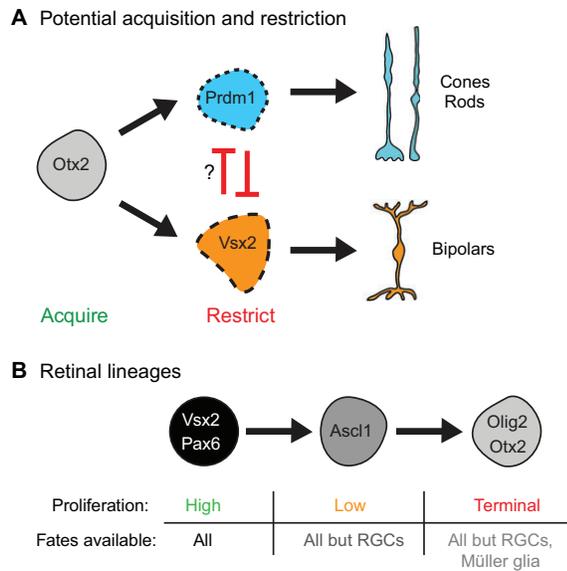
Fig. 2. Fate potential and lineage relationships in the mouse retina.
(A) A large fraction of precursors express Otx2 and acquire the potential to become photoreceptors (rods or cones) and bipolar cells. Otx2, in turn, activates the expression of Prdm1 and Vsx2, which may restrict bipolar and photoreceptor potential, respectively. (B) A population of progenitors (expressing Vsx2 and Pax6) that can give rise to all retinal cell types is present during embryogenesis. Retroviral lineage-tracing studies suggest that some of these progenitors have high proliferative ability. A large subset of progenitors expresses Ascl1 and loses the potential to form retinal ganglion cells (RGCs). Expression fate mapping suggests that these Ascl1-expressing progenitors have low to moderate proliferative ability. Progenitors that express Olig2 and/or Otx2 are in their terminal cell cycle and generate all but RGCs and Müller glia. Thus, Otx2 activation might be a default outcome for progenitors undergoing their last division.

et al., 2012). This suggests a model in which the initial progenitor pool produces precursors that express Atoh7, and these undergo subsequent specification events to produce ganglion cells. Those progenitors that remain in the cell cycle will express Ascl1 and generate Otx2-expressing precursors when they exit the cell cycle (Fig. 3A). Inhibition of Notch signaling seems to be the event that triggers cell cycle exit and the expression of Atoh7 at early stages of retinal development, and then Otx2 at later stages (Nelson et al., 2006, 2007; Maurer et al., 2014).

Signaling molecules involved in photoreceptor fate specification

Because of their unique morphology and gene expression profiles, photoreceptors were among the few neuronal subtypes that could be identified reliably *in vitro*. This allowed for the identification of soluble regulators of photoreceptor cell fate specification. These early experiments provided evidence for both an intrinsic potential 'clock' and for cell-to-cell interactions that control photoreceptor fate. In addition, co-culturing cells from early and late stages of retinal development provided evidence that factors in the progenitor cell environment might influence their ultimate fate (Watanabe and Raff, 1990, 1992; Altshuler and Cepko, 1992; Reh, 1992; Belliveau and Cepko, 1999; Belliveau et al., 2000). In particular, it was shown that factors present in the postnatal rat or mouse retina promoted the development of rhodopsin-expressing cells in the cultures. These initial findings were followed by studies that aimed to define the 'rod-inducing' molecule in postnatal cultures, and eventually both taurine and retinoic acid were found to promote rhodopsin expression in cultures of retinal cells (Altshuler et al., 1993;

Kelley et al., 1994). Other soluble factors, such as activin and Shh, also promote rhodopsin expression in some *in vitro* assays (Levine et al., 1997, 2000; Davis et al., 2000). It is not currently known where in the process of photoreceptor specification these factors act, but understanding the regulation of photoreceptor development by soluble factors is important for the derivation of rods and cones from stem cell sources (Box 3).

In addition to these soluble factors, the Notch signaling pathway has also been shown to play a key role in regulating the transition from multipotent progenitor to photoreceptor precursor. Notch signaling among progenitors and postmitotic cells is known to maintain the progenitor pool in multiple neuroepithelia, including the developing retina (Perron and Harris, 2000; Nelson et al., 2007; Xiang, 2013; Cepko, 2014). An unknown process causes a subset of the progenitors to inactivate the Notch signal and initiate the precursor gene expression program (Fig. 3A,B). For photoreceptors, as noted above, one of the earliest signs of this transition is Otx2 expression; inhibition of Notch signaling causes the induction of Otx2 expression in most progenitors of the mouse retina (Jadhav et al., 2006; Nelson et al., 2006, 2007; Yaron et al., 2006), while conditional deletion of Notch pathway components reduces the number of progenitors and increases the number of both rod and cone photoreceptor precursors (Jadhav et al., 2006; Yaron et al., 2006; Riesenberg et al., 2009; Mizeracka et al., 2013). There are at least three non-exclusive ways that Notch signaling might inhibit photoreceptor genesis: (1) by controlling the fraction of progenitors that exit the cell cycle at any given point during development; (2) by regulating photoreceptor potential (i.e. Otx2 expression); and (3) by influencing fate choice within postmitotic Otx2-expressing precursors. Further studies are required to dissect the mechanisms by which Notch acts in this context, although it should be noted that the ability to control retinal progenitor proliferation and photoreceptor specification using small molecule inhibitors of the Notch pathway has proven useful in several stem cell protocols (Box 3).

Transcriptional networks regulating photoreceptor identity

Once progenitors leave the cell cycle and stably express Otx2, other factors must influence and stabilize the ultimate pattern of gene expression that is characteristic of photoreceptors. As noted above, photoreceptors are neurons that express a unique set of genes required for phototransduction. The mechanism by which a photoreceptor precursor acquires this unique gene expression profile has been the subject of considerable investigation. This process could occur as the result of: (1) positive acting factors that instruct a fate choice; (2) negative regulators that block competing fate choices; or (3) more likely a combination of both mechanisms.

Downstream of Otx2 is a related transcription factor, Crx, which is necessary for the expression of most photoreceptor genes (Fig. 3B,C) (Chen et al., 1997; Freund et al., 1997; Furukawa et al., 1997). Deletion of *Crx* in mice does not prevent photoreceptor specification like *Otx2* deletion, but instead leads to a dramatic reduction in the expression of photoreceptor genes. A comparison of Crx and Otx2 ChIP-seq datasets shows that their transcriptional targets largely overlap in the retina, although there are also some unique targets (Samuel et al., 2014). *Crx* is expressed in both rods and cones and, as a result, mutations in this gene can lead to diseases that affect one or both photoreceptor types, such as Leber's congenital amaurosis and cone-rod dystrophy (Box 2) (Freund et al., 1997; Swaroop et al., 1999; Tran and Chen, 2014).
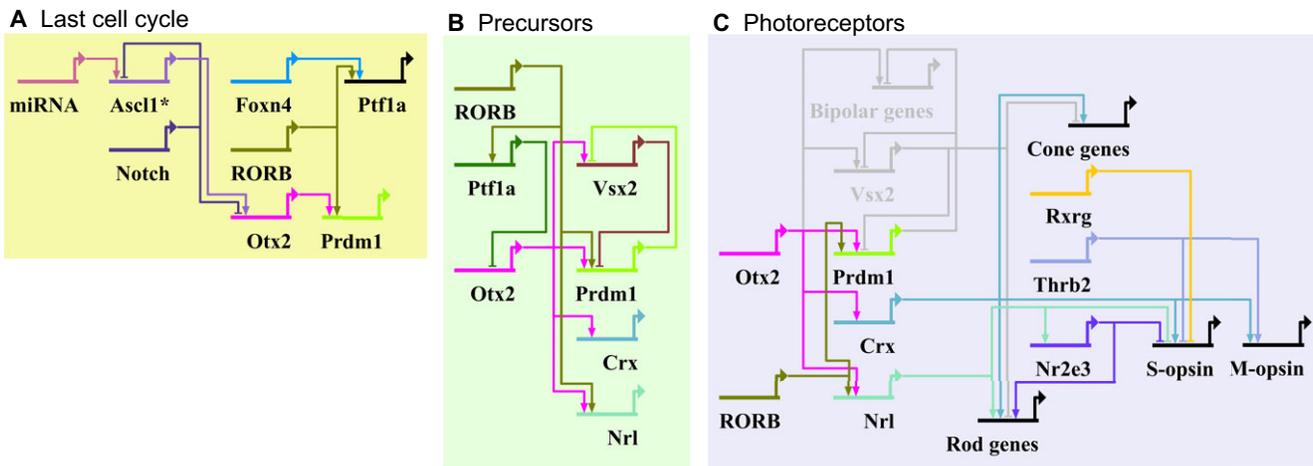
Fig. 3. Gene regulatory networks involved in photoreceptor development. (A) A possible gene regulatory network for progenitors in their last cell cycle. *Ascl1* can activate *Otx2*, although it is not necessary for this event. Thus, other factors (asterisk) and/or the loss of Notch signaling are needed to activate *Otx2* expression. The expression of *Ascl1* requires intact miRNA regulatory pathways. *Otx2* and *Rorb* combine to activate *Prdm1*. Alternatively, *Rorb* and *Foxn4* can cooperate to activate *Ptf1a* and push the cell towards amacrine or horizontal cell fates. Transient expression of *Otx2* and *Prdm1* does not prevent amacrine and horizontal development. (B) A possible gene regulatory network in Otx2-expressing precursors. *Ptf1a* suppresses *Otx2*, probably through downstream effectors. *Otx2* activates several genes needed for photoreceptor and bipolar cell formation. Many, perhaps all, of these targets require additional co-factors for their expression. *Prdm1* and *Vsx2* are likely to cross-repress each other to stabilize photoreceptor and bipolar fates, respectively. (C) A gene regulatory network that simultaneously shows the rod and cone specification pathways. *Otx2* and *Crx* are expressed by all photoreceptors. Downstream of *Otx2*, *Prdm1* silences the bipolar cell pathway (gray). *Nrl* and *Nr2e3* expression defines rods, whereas *Thrb2* and *Rxrg* expression marks cones. The activities of *Thrb2* (positive and negative) and *Rxrg* (negative) are needed to generate green (*Opn1mw*-expressing) cones. Drawn with BioTapestry (Longabaugh, 2012).

The distinction between rod and cone gene expression profiles occurs downstream (or independently) of Otx2 and Crx, and two key factors that control this specification event are Rorβ and Nrl (Swaroop et al., 2010). Otx2 and Rorβ are needed to initiate the expression of the transcription factor Nrl in a subpopulation of newly postmitotic precursors: these Nrl-expressing cells will develop into rod photoreceptors (Fig. 3C) (Akimoto et al., 2006; Kautzmann et al., 2011; Fu et al., 2014; Roger et al., 2014). Without either *Rorb* or *Nrl*, rod photoreceptor cells do not develop; instead, there is an approximately one-to-one fate shift to S-opsin-expressing cone cells (Mears et al., 2001; Fu et al., 2014). Nrl is needed to activate several essential rod-specific genes, including *Nr2e3*, a transcription factor that can both co-activate rod genes and silence cone genes, such as *Opn1sw* (S-opsin) (Fig. 3C) (Cheng et al., 2004, 2006, 2011; Chen et al., 2005; Peng et al., 2005; Hao et al., 2012). Accordingly, *Nr2e3* mutants exhibit excess S-opsin expression, reduced rod gene expression and significant rod defects (Akhmedov et al., 2000; Corbo and Cepko, 2005). When *Nrl* is overexpressed using a *Crx* enhancer (which drives expression in photoreceptors and bipolars), only cones, and not bipolar cells, become reprogrammed to a rod fate (Oh et al., 2007). Together, these data argue that achieving a normal rod photoreceptor gene expression profile requires the combination of rod activators (e.g. *Nrl*, *Nr2e3*) and a cone repressor (e.g. *Nr2e3*).

The factors that control the expression of these rod-specification factors are not clear, but are likely to exert their effects after the cells exit from the mitotic cycle in mice. This is because in lineage-tracing studies two-cell clones derived from Olig2^{+}, Neurog2^{+} and Ascl1^{+} progenitors sometimes contain two photoreceptors and sometimes a photoreceptor and a non-photoreceptor type (Brzezinski et al., 2011; Hafler et al., 2012). Live imaging experiments in zebrafish show that terminal divisions can generate similar diversity, although there are also lineage-restricted cone and rod progenitors (Godinho et al., 2007; He et al., 2012; Suzuki et al., 2013). These results imply that the specification events that define

rod and cone fates are not strictly linked to the status of the cell cycle. The data collected in mice suggest that a bipotential photoreceptor precursor is established shortly after cell cycle exit (Fig. 4A) (Swaroop et al., 2010). In this model, only these precursors can respond to Nrl and adopt rod fate. Several lines of evidence support this bipotent precursor model: (1) some rod and cone marker [i.e. Nrl and Thrβ2 (TRβ2)] overlap has been observed during development (Ng et al., 2011); (2) *Nrl* expression in developing cones can partially convert cells to a rod identity (Oh et al., 2007); and (3) removing *Nrl* from adult rods can partially convert them into cones (Montana et al., 2013). An alternative explanation (Fig. 4B) is that another factor causes progenitor cell commitment (see Glossary, Box 1) to rod identity upstream of Nrl, but after cell cycle exit (Emerson et al., 2013). In this model, Nrl would repress cone development and execute rod physiological maturation in photoreceptor precursors. The existence of a specification event prior to Nrl expression would account for the observation that photoreceptors are still specified in *Nrl* mutants. This would also explain why *Nrl* cannot reprogram all Crx-expressing cells into rods and why the cones seen in *Nrl* mutants are not identical (Daniele et al., 2005) to wild-type S-cones. Future work is needed to identify the earliest events in photoreceptor specification and discriminate between these two models.

Although a great deal of progress has been made in understanding the transcriptional network involved in rod photoreceptor specification, much less is known about how cones are committed to their fate. Only a few cone-specific markers have been characterized during embryonic retinal development. One such factor is the thyroid hormone receptor Thrβ2. Cones in mice are divided into blue (short wavelength) or green (medium wavelength) subtypes based on whether S-opsin (*Opn1sw*) or M-opsin (*Opn1mw*) is predominantly expressed, respectively. In contrast to human cones that predominantly express one opsin (blue, green or red) per cone, most mouse cones co-express both opsins in opposing dorsal-ventral gradients, such that M-opsin is highest dorsally and

Box 3. Photoreceptor development in vitro: the promise of stem cells

Protocols for the directed differentiation of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) into retinal cells have been developed and refined to the point where investigators can now generate laminated human or mouse retinal tissue. The initial protocols, using either mouse or human ESCs, used combinations of signaling molecules to anteriorize embryoid bodies prior to expansion as dissociated cell cultures (Ikeda et al., 2005; Lamba et al., 2006; Osakada et al., 2008, 2009). These protocols allow for large-scale expansion of retinal neurons. However, the production of photoreceptors under these conditions is relatively low. By contrast, protocols that maintain cells as embryoid bodies for extended periods allow for the development of highly organized, laminated retina-like structures in vitro (Eiraku et al., 2011; Lamba and Reh, 2011; Nakano et al., 2012; Phillips et al., 2012; Zhong et al., 2014). In these structures, the percentage of photoreceptors that develop is much higher than in dissociated cultures, suggesting that tissue organization is important for photoreceptor specification. In either case, both rod and cone photoreceptors develop and, in general, more cones are produced in human ESC-derived retinal cells than in mouse-derived cells (Nakano et al., 2012). Interestingly, the timing and sequence of neurogenesis are preserved in stem cell-derived retinas, as are the rate differences between mouse and human (Meyer et al., 2009). When ESC-derived retinal cells are transplanted, they can more fully differentiate into mature rods than they do in vitro. For example, both mouse and human ESC-derived photoreceptors develop morphological features of outer segments when transplanted into normal mouse retinas, and there is evidence that ESC-derived photoreceptors can respond to light (Lamba et al., 2009; Zhong et al., 2014).

Protocols to generate photoreceptors include both retinoic acid and taurine, which are two key factors that promote rod development (Altshuler et al., 1993; Kelley et al., 1994, 1999; Hyatt et al., 1996). Thyroid hormone can also promote cone photoreceptor development (Kelley et al., 1995) and it may promote red/green cone formation within human ESC-derived photoreceptors. The inhibition of Notch signaling with γ-secretase inhibitors, which drive retinal progenitors to exit the cell cycle both in the normal retina and in stem cell-derived cultures (Nelson et al., 2007; Lamba et al., 2009; Osakada et al., 2009), can also increase the number of photoreceptors generated.

S-opsin ventrally (Applebury et al., 2000; Swaroop et al., 2010). Mice deficient for *Thrb2* lack M-opsin expression and instead robustly express S-opsin in all cones, indicating a loss of the green cone subtype (Ng et al., 2001; Roberts et al., 2006). However, *Thrb2* null mice still generate cones, demonstrating that *Thrb2* is not required for commitment to cone identity. *Thrb2* is not sufficient for cone genesis either, as its expression in place of the *Nrl* gene in mice does not promote cone formation or prevent rod development (Ng et al., 2011). The transcription factor Rxrγ (*Rxrg*) is also expressed in developing cones and ganglion cells (Mori et al., 2001). As with *Thrb2* mutants, loss of *Rxrg* has no effect on the initial formation of cones (Roberts et al., 2005). M-opsin is expressed relatively normally, but S-opsin is robustly expressed by all cones in *Rxrg* mutants, disrupting subtype identity. Therefore, it appears that green cone subtype development is very similar to rod formation, requiring the action of a positive factor (i.e. *Thrb2*) to express M-opsin and the negative activities of both *Thrb2* and *Rxrg* to suppress S-opsin and blue cone identity (Fig. 3C).

How then are cones committed to their identity? There are three possibilities. First, and as discussed above, cone commitment might be the default outcome in the retina, requiring only negative regulators, such as Nr2e3 or Notch signaling, to generate non-cone fates (Fig. 4A,C). Second, it is possible that a cone-specific factor triggers commitment to a cone fate and/or suppresses rod fate,

analogous to Nrl (Fig. 4D). The identity of such a factor(s) remains unknown. Third, it is possible that the combinatorial action of more widely expressed regulators (i.e. not lineage restricted to photoreceptor fate) promotes cone commitment (Fig. 4E). A potential regulator is the transcription factor Onecut1, which is expressed in subsets of progenitors and Otx2-expressing cells, and later in horizontal and ganglion cells (Wu et al., 2012, 2013; Emerson et al., 2013). The forced expression of *Onecut1* results in the formation of excess cones at the expense of rods (Emerson et al., 2013). It was also shown that *Onecut1* and *Otx2* synergize to activate *Thrb2* expression, suggesting that the combinatorial action of these two factors is necessary for the acquisition of cone potential or cone fate commitment. In mice that lack *Onecut1* and its similarly expressed paralog *Onecut2*, there is an initial lack of Rxrγ-expressing cones, but the number of cones is only modestly reduced by the end of development (Sapkota et al., 2014). In addition, nearly all cones in these mutants express high levels of S-opsin and few cones express M-opsin, similar to *Thrb2* mutant mice. Thus, the combinatorial action of *Onecut1/2* and *Otx2* is not necessary for cone potential or commitment, but is needed for normal M-cone subtype development. Several other transcription factors that intersect early with Otx2, such as Neurod1, Ascl1, Neurog2 and Olig2, have been identified, although these do not prevent cone development when mutated (Tomita et al., 1996; Morrow et al., 1999; Hufnagel et al., 2010; Brzezinski et al., 2011; Hafler et al., 2012). However, it should be noted that mice lacking *Neurod1* fail to express *Thrb2* and have cone subtype defects (Liu et al., 2008). A model of cone commitment via the intersection of transcription factors and signaling cascades may therefore contain redundant components or compensatory mechanisms. The identification of additional early cone-specific markers and complex multi-factor gain- and loss-of-function studies are needed to distinguish between these three mechanisms of cone commitment.

Conclusions

Multiple experimental paradigms and animal model systems have expanded our knowledge of photoreceptor development, but significant gaps remain. Our understanding of early events in photoreceptor genesis, such as the regulation of potential and cone fate commitment, remains conspicuously incomplete. There is also a significant gap in our understanding of how intrinsic and extrinsic factors combine to regulate photoreceptor development both *in vivo* and in stem cell-derived cultures. The success of future regenerative therapies, such as cell replacement or endogenously stimulated regeneration, is highly dependent upon gaining a better understanding of photoreceptor fate specification.

Several studies have shown that retinal progenitors are multipotent and that their potential changes over developmental time. These data indicate that progenitors form a heterogeneous population, and recent experiments have shown that this heterogeneity can correlate with differential proliferative and fate outcomes (Brzezinski et al., 2011; Hafler et al., 2012). Furthermore, it has been shown that cells with the potential to form photoreceptors can also have the potential to form horizontal, amacrine and/or bipolar cells (Hafler et al., 2012; He et al., 2012; Brzezinski et al., 2013; Emerson et al., 2013). Does this mean that individual progenitors have the potential for all of these options simultaneously, or are cells subdivided into a series of states that have the potential for one or two fates at a time? In the latter case, progenitors and precursors might exist in a bistable gene expression state where one competing network becomes more
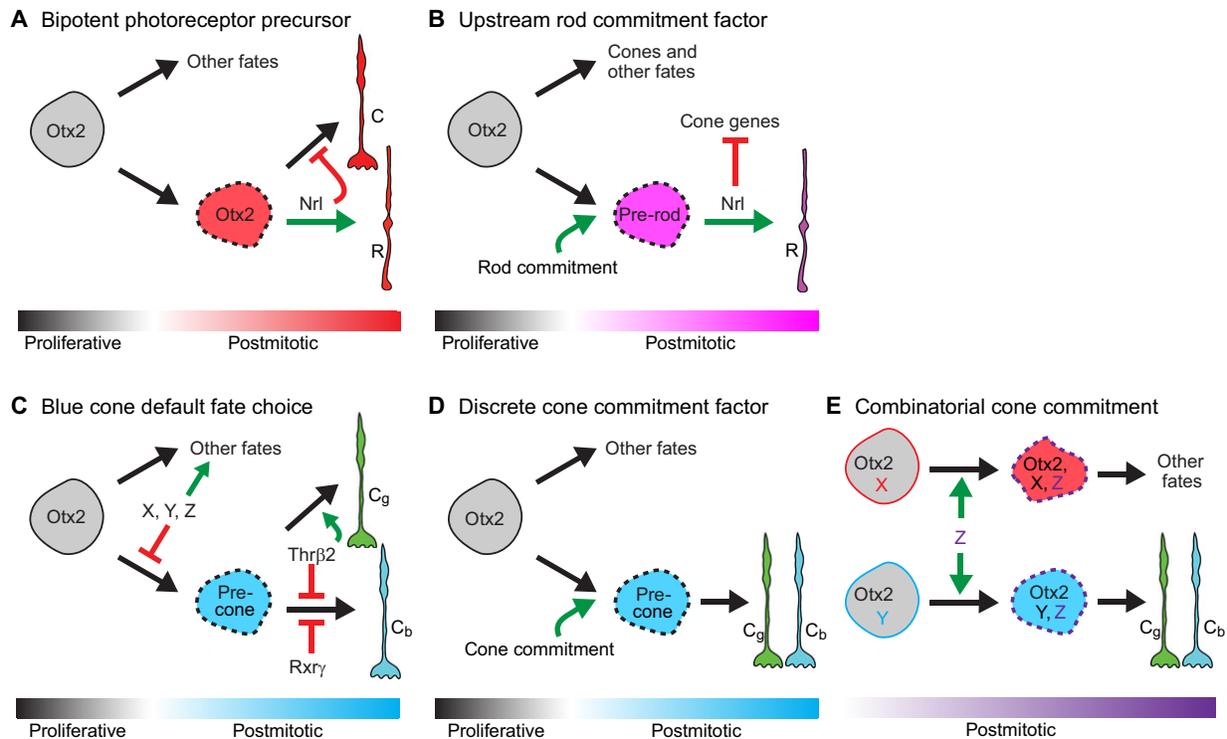
Fig. 4. Models of photoreceptor fate specification. (A) A bipotent Otx2-expressing precursor will become a cone (C) by default unless Nrl commits the cell to rod (R) fate. (B) Alternatively, cells might be committed to rod fate upstream of Nrl. In this case, Nrl would promote rod maturation while suppressing cone-specific genes. (C) Blue (*Opn1sw*-expressing) cone (C_b) fate might be the default outcome of Otx2-expressing cells. In this model, additional factors (X, Y, Z) are needed to suppress blue cone fate and promote other fates including green (*Opn1mw*-expressing) cones (C_g). (D) Cone development might not be the default outcome, but instead may require the action of a discrete commitment factor(s) that is expressed only by cones. (E) Cone commitment might be the result of the rare intersection of widely expressed (i.e. not restricted to photoreceptors) factors. In this example, Otx2-expressing precursors exposed to the same signal (Z) adopt different fates depending upon whether they co-express factor X or factor Y.

stabilized and 'wins', thereby changing the potential or committing the fate of the cell. It is unclear how many discrete bistable states a cell might experience to become specified as a photoreceptor or whether the path to a stable photoreceptor gene expression network is invariant. Also of importance is how potential is suppressed in progenitors and postmitotic cells. Recent data suggest that *Prdm1* restricts bipolar cell potential and that, in the absence of this inhibition, photoreceptor cell fate is mutable (Brzezinski et al., 2013). Does this type of fate plasticity exist more broadly in the retina? Further experiments are needed to test how providing potential and preventing potential restriction alters gene expression networks (i.e. fate outcomes) in both the developing and mature retina.

We have also learned much about rod development and cone subtype choice, but how cells are committed to cone identity is unclear. Although Thrβ2 and Rxrγ are useful for marking developing cones, the earliest marker of an irreversibly committed cone photoreceptor is yet to be identified. Further work is needed to discover whether cones: (1) are the default outcome of not becoming a rod photoreceptor; or (2) are actively committed from cells that have photoreceptor potential. In either case, it is unknown why some cells decide to activate *Nrl* and adopt rod identity whereas others commit to cone fate. Since the rod-to-cone ratio varies across vertebrate species, comparative developmental studies are likely to help unravel cone fate choice mechanisms.

In recent years, much effort in the field has been focused on generating retinal cells from embryonic and induced pluripotent stem cell sources (Box 3). Success generating retinal progenitors from these sources has been achieved by recapitulating the early

events of eye development using small molecules and growth factors (Lamba et al., 2008). The relative ease of using extrinsic factors to program stem cell fate has fueled renewed interest in understanding how these molecules could be used to directly control photoreceptor development. For example, pro-rod factors identified in dissociated culture systems (e.g. taurine and retinoic acid) have been used to promote rod fate and maturation in stem cell-derived cultures (Zhong et al., 2014). Evaluating how extrinsic factors (whether native to the retina or not) control photoreceptor potential, commitment and maturation will facilitate the specific generation of rods and cones from stem cell cultures. Evidence suggests that the maturation state of donor photoreceptors strongly influences transplant success (MacLaren et al., 2006). Thus, using extrinsic factors to achieve a synchronous state of photoreceptor maturation is expected to significantly improve the efficacy of cell replacement approaches.

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