Intrinsic mechanisms that control the specification of mammalian retinal cell types

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Representative publications

Abstract
The generation of diverse neuronal types and subtypes from multipotent progenitors during development is a prerequisite for establishing functional neural circuits in the central nervous system (CNS). The retina has long served as an excellent model system in which to study the mechanisms of CNS neuronal specification and diversification due to its accessibility and relative simplicity. During mammalian retinogenesis, seven classes of cells are specified from multipotent progenitors by the action of various intrinsic and extrinsic factors. Recent molecular genetic studies involving loss-of-function and gain-of-function approaches have uncovered a number of transcription factors, in particular, several homeodomain and basic helix-loop-helix (bHLH) factors, as pivotal intrinsic regulators of mammalian retinogenesis. These factors are found to act at different developmental processes to establish progenitor multipotency, define progenitor competence, determine cell fates, and/or specify cell types and subtypes. These findings have provided an important framework for a full understanding of gene regulatory networks involved in the generation of neuronal diversity.

Introduction
The mammalian retina is a highly organized sensorineural tissue composed of six classes of neurons and one type of glial cells that are interconnected in a laminar structure. These include the rod and cone photoreceptor cells in the outer nuclear layer; the horizontal, bipolar, and amacrine interneurons plus the Müller glial cells in the inner nuclear layer; and the ganglion and displaced amacrine cells in the ganglion cell layer. In addition, a small number of displaced amacrine and ganglion cells are located in the inner plexiform layer. Apart from these seven major cell classes, some classes of retinal neurons contain many diverse subtypes that differ in morphologies, physiological properties and functions12.

Owing to its accessibility, well-characterized cell classes and neural tube origin, the retina has provided an excellent model system in which to study the mechanisms of neuronal determination and
differentiation in the CNS. During embryogenesis, the retina develops from the optic vesicle, a protrusion of the diencephalic neuroepithelium of the neural tube. A central question in retinal development is how those diverse retinal cell types and subtypes are specified and differentiated from an initially seemingly uniform neuroepithelium. Birthdating analysis by tritiated thymidine labeling has revealed a loose and yet fixed temporal order for the genesis of each cell type\textsuperscript{3,4} (Fig. 1). For instance, during murine retinogenesis, ganglion cells are the first cell type to exit the cell cycle, followed closely by horizontal, amacrine and cone cells, while rod, bipolar and Müller cells are the last cell types to be produced (Fig. 1). Despite this loose temporal order, multiple cell types can be generated at any given age of retinal development (Fig. 1). Moreover, lineage tracing studies have clearly demonstrated that retinal progenitors are multipotent, suggesting that retinal cells are determined without following strict cell lineages, but rather depending on local environment and cell-cell interactions\textsuperscript{5-8}. However, intrinsic properties of progenitors must also contribute to the choice of cell fate since the progenitors must first become competent to respond to extrinsic cues to generate appropriate cell types. In this review, we will focus on recent advances in the identification of intrinsic transcriptional regulators that control fate commitment and differentiation of various retinal cell types and subtypes.

**Current model of retinal cell specification**

As discussed above, there exists some inconsistency between the results obtained from birthdating and lineage tracing studies. To accommodate these sometimes contradictory findings, a recent model of retinogenesis suggests that intrinsic and extrinsic factors together determine the choice of cell fate\textsuperscript{9-11}. In this model, it is hypothesized that during retinogenesis, progenitors pass through
successive and distinct states of competence to respond to temporally varying environmental cues for the ordered generation of different cell types (Fig. 2). Each state of competence is transient and probably defined by a network of transcription factors. The progression of competence states appears to be unidirectional and these states may not be skipped. Once committed by extrinsic cues, the progenitor cell moves forward to form a proper cell type following a particular differentiation program.

Fig. 2. Model of retinal development. Retinal progenitors may undergo a series of changes in competence to respond to environmental cues for producing different cell types. Each progenitor cell is thought to be controlled by a network of transcription factors that define its competence to make a particular retinal cell type, or a small set of cell types. Retinal progenitors may progress from one state of competence to another only in one direction. Early progenitor cells may be unable to jump ahead to later stages of competence. The commitment of a competent cell to become a particular cell type is controlled by extrinsic signals. Once committed, the progenitor differentiates into the appropriate cell type.

This model can elegantly reconcile most of the data accumulated to date on retinogenesis as well as can nicely explain the ordered and yet overlapping phases of generation of different cell types. However, one should keep in mind that this is a working model that is still evolving as new data emerge. For instance, a recent report suggests that the progression of competence states may be reversible since a subset of late progenitors can generate early born ganglion cells at conditions conducive for their differentiation\(^{12}\). Furthermore, the current model does not consider the relative importance of intrinsic and extrinsic factors in a particular cell fate decision or deal with the mechanism regulating the total number of each cell type generated. There is currently limited evidence implicating intrinsic factors as the major determinants of cell fate choice in the developing retina\(^{13}\).

**Transcriptional regulators as intrinsic factors controlling retinal development**

Retinal development involves both intrinsic and extrinsic factors for cell fate commitment and differentiation. It is generally assumed that a competence state of progenitor cells is defined by the
expression of a particular combination of transcription factors. Similarly, extracellular signals may affect the choice of cell fate by programming the expression and activity of transcription factors in competent progenitors. The transcription factors in turn modulate the expression of specific sets of target genes, which ultimately specify cell fates and program differentiation. Therefore, transcription factors play essential roles in controlling cell determination and differentiation during retinogenesis. To identify transcription factors and subsequently elucidate their functions in the retina will be an important first step toward understanding the molecular events that lead to this highly specialized sensorineural tissue. In the past decade or so, experiments that perturb normal expression of transcription factor genes have provided fundamental insights into the molecular mechanisms of cellular fate commitment and differentiation during retinogenesis. A variety of transcription factors have been found to control intrinsic properties of retinal progenitors and/or participate in differentiation processes. These include two classes of factors, the homeodomain and bHLH transcription factors that have emerged as some of the major molecular players.

**Multipotent state of progenitors.** Prior to the initiation of retinogenesis, neuroepithelial cells in the optic vesicle must first acquire and then maintain multipotency for the generation of the full range of retinal cell types. How this comes about still remains to be determined but two transcription factors, Pax6 and Foxn4, have been demonstrated to play a crucial role in this process (Figs. 3, 4). Pax6 is a paired-type homeodomain factor that is required for patterning early eye development. Its mutations or overexpression result in a range of ocular phenotypes including small eyes, absence of eyes, cataract, or aniridia in the mouse or human\(^{14-18}\). Although Pax6 is expressed by all retinal progenitor cells, its essential early patterning role has made it difficult to gauge a possible later function during retinal development. The advent of the conditional gene knockout technique has resolved this problem and we now know that all retinal cell types except for γ-aminobutyric acid (GABA)ergic amacrine cells disappear when Pax6 is ablated specifically from retinal progenitor cells\(^{19}\). Thus, Pax6 appears to be required by retinal progenitors to acquire and/or maintain the multipotent state. Moreover, it appears to do so by regulating the expression of multiple retinogenic bHLH factors which are key intrinsic regulators controlling cell type specification\(^{19,20}\).

Foxn4, a winged-helix/forkhead transcription factor that is expressed by a large subset of retinal progenitors, appears to be required by progenitors to acquire competence states for the generation of amacrine and horizontal cells\(^{21}\). Loss of Foxn4 function in mice causes a loss of all horizontal cells as well as the great majority of amacrine cells, while overexpression of Foxn4 biases most progenitors into an amacrine cell fate. Similar to Pax6, Foxn4 appears to control the genesis of amacrine and horizontal cells by activating retinogenic bHLH and homeodomain factors that are involved in the specification of these two cell types\(^{21}\). It is conceivable that Pax6 may act together with Foxn4 and other homeodomain and bHLH factors to confer retinal progenitors with the full potential for the genesis of all seven cell classes (Figs. 3, 4).
Fig. 3. Transcription factors involved in the specification and differentiation of different retinal cell types.

Fig. 4. Mechanism that controls the specification of amacrine and horizontal cells by retinal progenitors. Retinal progenitors with the activation of both Foxn4 and Pax6 (Pax6\(^+\)Foxn4\(^+\)) are competent for the genesis of all horizontal cells and the great majority of amacrine cells. Only a small number of amacrine cells are generated from the Pax6\(^+\)Foxn4\(^-\) progenitors. Some of the newly generated amacrine cells may differentiate into glycineceric cells by the action of Barhl2 and Pax6. Foxn4 confers progenitors with the competence for the genesis of amacrine and horizontal cells by activating the expression of Math3, NeuroD1 and Prox1, three retinogenic factors involved in the specification of these two cell types. The rod, cone, bipolar, ganglion, and Müller cells are likely to be derived largely from the Pax6\(^+\)Foxn4\(^+\) progenitors via activation of other retinogenic factors\(^{21,52}\).

**Photoreceptors.** Although retinal progenitor cells can exist in multiple competent states as development progresses, they require the function of particular intrinsic factors to commit to a particular cell fate. For photoreceptor cells, two homeodomain transcription factors, Otx2 and Crx,
are required for fate commitment and differentiation, respectively (Fig. 3). Conditional gene targeting of Otx2 results in a complete absence of rod and cone cells while causing a fate-switch in progenitors to generate more amacrine cells; whereas its overexpression promotes a photoreceptor cell fate\(^22\). Otx2 controls photoreceptor development partly by activating the expression of Crx\(^22\), which has been shown by gene targeting and overexpression analyses to be essential for differentiation but not for determination of photoreceptor cells\(^23,24\). Microarray and other studies have revealed that Crx programs photoreceptor differentiation by regulating the expression of a network of photoreceptor-specific/enriched genes. These include genes involved in the phototransduction pathway such as those encoding opsins, transducins, phosphodiesterases, and arrestins\(^24,25\). In the human, mutations in Crx are associated with retinal diseases including cone-rod dystrophy, retinitis pigmentosa, and Leber congenital amaurosis\(^26-29\). As also demonstrated by gene targeting and overexpression experiments, the terminal differentiation and survival of photoreceptors require the critical function of another transcription factor, the NeuroD1 bHLH factor\(^30,31\) (Fig. 3).

The mechanism of photoreceptor subtype specification remains to be defined but thus far three key intrinsic transcription factors have already been identified that play such a role. These are the Nrl basic motif-leucine zipper (bZIP) factor, the Nr2e3 orphan nuclear receptor, and the thyroid hormone receptor 2 (Tr 2) (Fig. 3). In the human, missense mutations in NRL are associated with autosomal dominant retinitis pigmentosa\(^32,33\). In the mouse, the absence of Nrl leads to a switch in the rod fate\(^34\), generating supernumerary S (blue)-cones with resemblance to the symptom of enhanced S-cone syndrome (ESCS), which is caused by mutations in NR2E3 in humans\(^35\). A null Nr2e3 mutation in mice results in the rd7 retinal degeneration characterized by the abundant presence of a morphologically hybrid photoreceptor and a modest increase of S-cones\(^36-39\). Nr2e3 is expressed exclusively in rods and appears to influence rod differentiation primarily by repressing a large number of cone-specific/enriched genes in rods, as demonstrated by microarray and several other techniques\(^37-39\). Similar approaches have revealed that Nrl functions to determine rods from progenitor cells by activating numerous rod-specific genes as well as by suppressing cone-specific genes in part by regulating Nr2e3 expression\(^34,40\). Interestingly, both Nrl and Nr2e3 control downstream gene expression by interacting with Crx at their target DNA-binding sites\(^39,41\). The specification of M (green)-cones has been shown to be critically dependent on the function of Tr 2 as the absence of its function causes a complete loss of M-cones and a concomitant increase of S-cones\(^42\).  

**Bipolar cells.** Fate determination of bipolar cells relies on the synergistic activities between the Chx10 homeodomain factor and the two bHLH factors Mash1 and Math3 (Fig. 3). Mutations in Chx10 block bipolar cell specification as well as retinal progenitor proliferation, and have been shown to be responsible for the mouse mutation *ocular retardation*\(^43\). Retinas deficient for both Mash1 and Math3 lack bipolar cells and display a fate change to Müller cells\(^44,45\). However, albeit necessary, none of the three factors appear to be sufficient on their own for the selection of a bipolar fate.
When each of them is overexpressed in retinal progenitor cells, they do not exert any effect on bipolar cell formation. Only when Chx10 is co-expressed with Mash1 or Math3 are more bipolar cells formed, indicating that commitment to a bipolar cell fate requires a combinatorial action of Chx10 and Mash1 or Math3 in progenitor cells.

In the mouse retina, there exist one type of rod bipolar cells and at least nine types of morphologically and physiologically distinct cone bipolar cells. At present, it is entirely unclear how each of these subtype identities is specified but strides have been made to identify some of the intrinsic factors involved in their differentiation. For instance, the bHLH factor Bhlhb4 is expressed by all developing rod bipolar cells and loss of Bhlhb4 function causes a near complete loss of these cells due to a failure in their terminal differentiation. As for cone bipolar cells, two homeodomain factors Vsx1 and Irx5 are involved in the maturation of cone bipolar subtypes.

Amacrine and horizontal cells. The specification of amacrine cells involves the concerted activities of Foxn4 and NeuroD1 or Math3. As discussed above, Foxn4 is required by retinal progenitors to establish the competence state for the generation of amacrine cells. It appears to do so in part by activating the expression of Math3 and NeuroD1. In compound knockout mice deficient for both NeuroD1 and Math3, a complete loss of amacrine cells is accompanied by a fate-switch of progenitors to ganglion and Müller cells; whereas, the formation of amacrine cells is essentially normal in single mutants null for either NeuroD1 or Math3, indicating that NeuroD1 and Math3 are redundantly required for fate determination of amacrine cells. Overexpression experiments have shown that NeuroD1 alone or in combination with Pax6 is capable of promoting amacrine cell differentiation. Similarly, Math3 together with Pax6 can promote amacrine cell differentiation but Math3 alone lacks this activity. Therefore, besides its role in the establishment of progenitor multipotency, Pax6 may also be involved in the specification of amacrine cells even though it is not necessary for their generation. The absence of Pax6 has been shown not to alter NeuroD1 expression during retinogenesis, thereby allowing the differentiation of amacrine cells to occur. However, Pax6 and another homeodomain factor, Barhl2, are expressed by differentiating and mature amacrine cells and have been implicated as playing a role in the specification and/or differentiation of glycinergic amacrine cells (Figs. 3,4).

Despite our current knowledge of amacrine cell development, it remains to be determined what factors are involved in the specification of amacrine subtypes, numbered at least 26 in the mouse retina.

Only until recent 2-3 years have we begun to understand the intrinsic molecular basis of horizontal cell development. Similar to amacrine cells, Foxn4 is clearly required for the genesis of horizontal cells as retinas deficient for Foxn4 fail to generate any of them. However, contrary to its potent activity in the promotion of amacrine cell formation, Foxn4 is unable to promote horizontal
cell differentiation when overexpressed in progenitors. Thus, Foxn4 is required only for competence acquisition but not for fate specification of horizontal cells. On the other hand, Prox1, a homeodomain factor expressed by a small set of progenitors, has been shown to act as a crucial intrinsic factor that controls fate commitment of this cell type (Figs. 3, 4). Targeted Prox1 deletion results in loss of horizontal cells accompanied by a fate-switch of progenitors to rod and Müller cells while its overexpression strongly promotes a horizontal cell fate. Foxn4 appears to confer retinal progenitors with the competence for a horizontal cell fate in part by directly or indirectly activating Prox1 expression.

**Ganglion cells.** Two key intrinsic transcriptional regulators, Math5 and Brn3b, have been identified that control retinal ganglion cell development (Fig. 3). Math5 is a bHLH factor that is expressed by a subpopulation of progenitor cells and has been shown to confer progenitors with the competence for a ganglion cell fate. Mouse retinas deficient for Math5 fail to generate the great majority of ganglion cells and instead overproduce amacrine and cone cells. Since not all progenitors expressing Math5 are committed to a ganglion cell fate as demonstrated in a lineage study, Math5 is thought to be essential only for competence acquisition but not for fate commitment to ganglion cells. The intrinsic factor(s) that acts to specify ganglion cells from competent progenitors remains to be determined. Acting downstream from Math5, Brn3b is a POU-homeodomain transcription factor expressed by differentiating and mature ganglion cells. Loss of Brn3b function leads to a loss of most ganglion cells as a result of inappropriate early and terminal differentiation while its overexpression can weakly promote ganglion cell differentiation.

Microarray and other analyses have determined the network of genes regulated by Math5 and Brn3b during ganglion cell development. Both factors control the expression of a large set of downstream genes and the set downstream of Brn3b constitutes only one branch of the Math5 gene regulatory network. Notably, Math5 is found to activate Brn3b expression in differentiating ganglion cells, which in turn activates the expression of Brn3a and Brn3c, two Brn3b homologs with a role redundant with that of Brn3b during ganglion cell differentiation. The continued expression of Brn3b in differentiated ganglion cells appears to be maintained in part via feedback autoregulation and cross-activation by Brn3a and Brn3c (Fig. 3). Wt1, a zinc-finger transcription factor encoded by the Wilms’ tumor gene, appears to regulate ganglion cell differentiation also by directly activating Brn3b expression. However, the relationship between Math5 and Wt1 is not clear at present.

**Müller cells.** Thus far, several transcription factors have been identified that influence the specification of Müller glial cells (Fig. 3). Hes1, Hes5 and Hesr2 are three closely related bHLH transcriptional repressors whose expression is found early in retinal progenitors but later restricted to Müller cells. When overexpressed in progenitor cells, all three factors strongly promote a Müller cell fate at the expense of neurons. Conversely, loss of Hes5 function causes a decrease in the production of Müller cells. The paired-type homeodomain factor Rax
exhibits an expression pattern similar to that of the Hes factors and it too has a potent activity to induce Müller cell development. Rax may promote Müller cell formation in part by directly activating Hes1 gene expression.

Conclusion remarks

Recent years have witnessed rapid advances in our understanding of the intrinsic molecular mechanisms governing retinal cell type specification and differentiation. Molecular genetic studies involving loss-of-function and gain-of-function approaches have unraveled a number of transcription factors, in particular, several homeodomain and bHLH factors, as pivotal intrinsic regulators of mammalian retinogenesis. These findings have provided significant insights into the molecular bases that define progenitor competence states and program cell type specification. Despite these exciting advances, however, our understanding of the relationship between various intrinsic factors and the gene regulatory networks in which they participate still remain rudimentary. Continued microarray and other studies such chromatin immunoprecipitation promise to fill some of the knowledge gaps. Another issue is the intrinsic mechanism, of which little is known at present, that underlies subtype specification of different retinal cell classes. Given the extreme diversity of some cell classes such as amacrine and ganglion cells, the mechanism may prove to be exceedingly complex. For instance, there are at least 26 morphological subtypes of amacrine cells in the mouse retina. Research aimed at a molecular elucidation of the mechanism that specifies subtype identities such as these will undoubtedly lead to many more exciting discoveries in years to come.

As a crucial part of our visual system, the retina is not only scientifically intriguing but also clinically important. Identification and functional characterization of transcription factors involved in retinal development have provided fundamental insights into the molecular mechanisms that govern mammalian neurogenesis. In the long term, understanding the regulatory molecules involved in retinogenesis may provide the foundation for improved treatment of some blinding diseases, since defects in these molecules are often associated with retinal disorders in both human and experimental animal models. In this regard, mutations in several transcription factors cause eye abnormalities as described above. Furthermore, given the potential applications of retinal stem cells in treating retinal degenerative diseases, it is clearly of considerable interest to understand the molecular basis of normal retinogenesis so that we may better manipulate stem cells to achieve controlled regeneration of desired retinal cell types as a possible stem cell-based therapy.

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