Roles of homeobox and bHLH genes in specification of a retinal cell type

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SUMMARY

Previous analysis of mutant mice has revealed that the bHLH genes Mash1 and Math3, and the homeobox gene Chx10 are essential for generation of bipolar cells, the interneurons present in the inner nuclear layer of the retina. Thus, a combination of the bHLH and homeobox genes should be important for bipolar cell genesis, but the exact functions of each gene remain largely unknown. We have found that in Mash1-Math3 double-mutant retina, which exhibits a complete loss of bipolar cells, Chx10 expression did not disappear but remained in Müller glial cells, suggesting that Chx10 expression per se is compatible with gliogenesis. In agreement with this, misexpression of Chx10 alone with retrovirus in the retinal explant cultures induced generation of the inner nuclear layer-specific neuronal subtypes in the presence of Mash1-Math3, raising the possibility that the bHLH genes may be involved in neuronal subtype specification, in addition to simply making the neuronal versus glial fate choice.

Key words: bHLH, Bipolar cell, Chx10, Homeobox, Mash1, Math3, Retina, Mouse

INTRODUCTION

During neural development, precursor cells give rise to a wide variety of neurons and glial cells. It has been shown that combinations of transcription factors, such as homeobox and basic helix-loop-helix (bHLH) factors, are involved in specification of many neuronal subtypes (for review, see Tanabe and Jessell, 1996; Anderson and Jan, 1997; Goridis and Brunet, 1999; Guillemot, 1999). For example, homeobox genes such as Otx1 and Otx2, and the bHLH genes such as neurogenin 1 (Ngn1; Neurod3 – Mouse Genome Informatics) and Ngn2 (Atoh4 – Mouse Genome Informatics) regulate neuronal development in the brain (Acampora and Simeone, 1999; Fode et al., 2000). In Otx1-Otx2-mutant mice, rostral-type neurons are transformed into more caudal-type neurons (Acampora et al., 1997; Suda et al., 1997) while, in Ngn1-Ngn2-mutant mice, dorsal-type neurons are transformed into ventral-type neurons (Fode et al., 2000), indicating that combinations of homeobox and bHLH factors play an important role in neuronal subtype specification in the brain. However, it remains to be determined how these different classes of transcription factors coordinate the processes of neuronal subtype specification.

Retina is an ideal model system in which to analyze the molecular mechanism of neural development, as it comprises only six types of neurons and one type of glia, all of which can be identified by their position, morphology and specific markers (Cepko, 1999). These seven types of cells constitute three cellular layers in the retina: the outer nuclear layer (ONL), which contains rod and cone photoreceptors, the inner nuclear layer (INL), which contains bipolar, horizontal and amacrine interneurons, and Müller glia, and the ganglion cell layer, which contains ganglion cells (projection neurons). These retinal cells differentiate from common precursors with different time courses. It has been shown that many transcription factors regulate specification of retinal cell types. For example, bHLH genes such as Mash1 (Ascl1 – Mouse Genome Informatics) and NeuroD (Neurod – Mouse Genome Informatics) not only promote neuronal versus glial fate determination but also regulate the neuronal subtype specification (Kanekar et al., 1997; Morrow et al., 1999; Cepko, 1999). In addition to bHLH genes, homeobox genes such as Crx are also required for specification of retinal cell types (Furukawa et al., 1997; Furukawa et al., 1999; Freund et al., 1997; Chen et al., 1997). However, it still remains to be determined whether the two classes of transcription factors, bHLH and homeobox factors, have different or overlapping functions in retinal cell fate specification.
Recent studies have demonstrated that bipolar cell development is also regulated by at least two different classes of transcription factors. Both the homeobox gene 

**Math3**

and the bHLH genes **Mash1** and **Math3** (Atoh1 – Mouse Genome Informatics) are expressed initially by retinal precursor cells and then by bipolar cells (Liu et al., 1994; Jasoni and Reh, 1996; Takebayashi et al., 1997; Roztocil et al., 1997). Mutation of **Chx10** results in reduction of retinal precursors and a complete loss of bipolar cells (Burmeister et al., 1996). Likewise, although there is no abnormality in **Math3** mutant embryos, harvested at E15.5. but died by E17.5, embryos were mutant embryos survived until E15.5 but died by E17.5, embryos were completely missing and instead Müller glial cells are significantly increased (Tomita et al., 1996b; Tomita et al., 2000). Thus, bipolar cells are lost in both **Chx10** and **Mash1-Math3** mutations, demonstrating that the two classes of transcription factors are essential for specification of the bipolar cell fate. In spite of these similar phenotypes, a concomitant increase of the Müller glial cell number observed in **Mash1-Math3** double-mutant retina does not occur in **Chx10-deficient retina**, and therefore the exact phenotypes are different between **Chx10** and **Mash1-Math3** mutations, suggesting that the two classes of factors have distinct roles in specification of the bipolar cell fate.

We have found that in **Mash1-Math3** double-mutant retina, **Chx10** expression did not disappear in spite of a complete loss of bipolar cells, but remained in Müller glial cells. To further decipher the functions of different classes of transcription factors in bipolar cell genesis, each gene was misexpressed with retrovirus in the developing retina. Interestingly, misexpression of **Chx10** predominantly generated INL-specific cells, including Müller glia, but was not sufficient for generation of mature bipolar cells, suggesting that **Chx10** confers the INL-specific identity but not the neuronal versus glial lineage information. Misexpression of **Mash1** or **Math3** alone did not induce bipolar cells either but inhibited Müller glial development. In contrast, misexpression of **Chx10** together with **Mash1** or **Math3** promoted bipolar cell genesis and inhibited gliogenesis. These results indicate that the homeobox and bHLH genes confer the layer identity and the neuronal versus glial lineage information, respectively, and together specify a neuronal subtype in the retina. Moreover, **Mash1** and **Math3** promoted the bipolar cell fate but not the other INL-specific neuronal subtypes in the presence of **Chx10**, raising the possibility that the bHLH genes may be also involved in neuronal subtype specification in addition to simply making the neuronal versus glial fate choice.

**MATERIALS AND METHODS**

**Mash1-Math3 mutant mice**

**Math3** mutant mice were generated by replacing the majority of the coding region with the neo cassette (Tomita et al., 2000). **Mash1-Math3** double-mutant mice were obtained by crossing **Mash1**–**Math3** male and **Mash1**–**Math3** female mice. Because double-mutant embryos survived until E15.5 but died by E17.5, embryos were harvested at E15.5.

**Construction of retroviruses**

For CLIG-Mash1, CLIG-Math3, CLIG-Ngn2, CLIG-Chx10 and CLIG-Pax6, cDNAs for bHLH and homeobox factors (with or without three copies of the Myc epitope tag) were cloned into the EcoRI site of pCLIG, which directs expression of the cloned genes together with enhanced green fluorescent protein (GFP) from the upstream long terminal repeat (LTR) promoter with cytomegalovirus enhancer (Hojo et al., 2000). For CLIG-Chx10-Mash1, CLIG-Chx10-Math3 and CLIG-Chx10-Hes5, the bHLH genes were cloned into the BsrGI and ClaI sites of pCLIG-Chx10, which are located in the 3′ region of GFP gene, so that GFP gene is fused in frame with each bHLH gene. Retroviral DNAs were transfected with LipofectAMINE (Gibco-BRL) into Ψ2mp34, an ecotropic packaging cell line (Yoshimatsu et al., 1998). The supernatant was collected two days later and concentrated with Centricon Plus-20 (Millipore), as described previously (Ishibashi et al., 1994; Tsuda et al., 1998). 10 μl of the virus solution was applied to the retinal explant cultures. For the clonal size analysis, only 1 μl was applied to identify clones.

**Retinal explant culture**

The retinal explant culture was performed as described previously (Tomita et al., 1996a). Briefly, eyes were isolated from E15.5 or E17.5 mouse embryos and transferred to PBS solution. The neural retina without pigment epithelium was placed on a Millicell chamber filter (Millipore; diameter 30 mm, pore size 0.4 μm) with the ganglion cell layer upwards. The chamber was transferred to a six-well culture plate. Each well contained 1 ml of culture medium (50% MEM with Hepes, 25% Hank’s solution, 25% heat-inactivated horse serum, 200 μM L-glutamine and 5.75 mg/ml glucose). Explants were cultured at 34°C in 5% CO₂, and the medium was changed every other day.

**Dissociation of retinal cells and immunocytochemistry**

For quantification of virus-infected cells, retinæ were dissociated after two weeks of culture, as previously described (Morrow et al., 1998). Briefly, neural retinae were incubated with 0.05% trypsin in HBSS lacking Ca²⁺/Mg²⁺ for 10 minutes at 37°C and then two and a half times volume of the culture medium containing 10% FBS was added. The cells were pelleted, resuspended in HBSS containing 20 μg/ml DNaseI, triturated and plated on poly-D-lysine-coated eight-well glass slides (Nalge Nunc). After incubation for 3 hours at 37°C in 5% CO₂, the cells were fixed with 4% paraformaldehyde for 10 minutes on ice and subjected to immunocytochemical analysis.

**Immunocytochemical analysis**

After 2 weeks of culture, retinal explants were fixed with 4% paraformaldehyde for 10 minutes on ice, treated with 25% sucrose for 30 minutes, embedded in OCT compound (Miles), and sectioned (16 μm thickness). For immunocytochemistry, sections or dissociated cells on slides were preincubated with a blocking solution (5% normal goat serum and 0.1% Triton X-100 in PBS) for 1 hour, and then incubated overnight at room temperature or for two days at 4°C in 1% goat serum and 0.1% Triton X-100 in PBS) for 1 hour, and then incubated overnight at room temperature or for two days at 4°C in 1% goat serum and 0.1% Triton X-100 with the following antibodies (dilution rate): rabbit anti-GFP (1:300; Medical and Biological Laboratories), rabbit anti-Myc (1:1000; Medical and Biological Laboratories), mouse anti-calbindin (1:200; Sigma), mouse anti-protein kinase C (PKC) (1:100; Amersham), rabbit anti-rhodopsin (1:2000; LSL), mouse anti-glutamine synthetase (GS) (1:500; Chemicon), mouse anti-nestin (1:500; Pharmingen), rabbit anti-neuron-specific enolase (NSE) (1:1; Immunotech), rat anti-cyclin D1 (1:200; Calbiochem), mouse anti-cyclin D3 (1:100; Medical and Biological Laboratories), mouse anti-calbindin (1:200; Sigma), mouse anti-protein kinase C (PKC) (1:100; Amersham), rabbit anti-rhodopsin (1:2000; LSL), mouse anti-glutamine synthetase (GS) (1:500; Chemicon), mouse anti-nestin (1:500; Pharmingen), rabbit anti-neuron-specific enolase (NSE) (1:1; Immunotech), rat anti-cyclin D1 (1:200; Calbiochem), mouse anti-cyclin D3 (1:100; Santa Cruz), mouse anti-Ki67 (1:200; Pharmingen), mouse anti-Mash1 (1:30; Pharmingen) and mouse anti-Pax6 (1:30; Hybridoma Bank). To detect these antibodies, FITC-conjugated anti-mouse IgG (1:200, Vector), FITC-conjugated anti-rabbit IgG (1:200, Vector), Cy3-conjugated anti-mouse IgG (1:200; Amersham), biotinylated anti-rabbit IgG (1:200; Vector), biotinylated anti-mouse IgG (1:200; Vector), FITC-avidin D (1:1000; Vector) and Texas Red avidin D (1:1000; Vector) were used. Retinal cell types were determined by the morphology, location and the following antibodies: anti-PKC, anti-NSE (bipolar cells), anti-calbindin (horizontal and amacrine cells), anti-GS, anti-cyclin D3 (Müller glia),...
anti-rhodopsin (rods), anti-nestin and anti-cyclin D1 (precursors). TUNEL assay was performed with a detection kit (Boehringer-Mannheim). Fluorescently labeled preparations were imaged using a Carl Zeiss confocal microscope.

**In situ hybridization**

In situ hybridization on frozen sections was performed as previously described (Hojo et al., 2000). Antisense strand probe of mouse Chx10 (Liu et al., 1994) was labeled with digoxigenin.

**RESULTS**

**Chx10 expression remains in Müller glia of Mash1-Math3 double-mutant retina**

We have recently found that, in Mash1-Math3 double-mutant retina, the cells that normally differentiate into bipolar cells adopt the Müller glial cell fate (Tomita et al., 2000). To determine whether this defect is due to a loss of Chx10 expression, we first performed in situ hybridization. At E15.5, both wild-type and Mash1-Math3 double-mutant retinæ consist of two cellular layers, the ganglion cell layer and the ventricular zone, which contains retinal precursor cells. Chx10 expression, which is observed in retinal precursor cells at this stage, was not altered in the double-mutant retina (data not shown). To examine a later stage of Chx10 expression, we next performed retinal explant culture prepared from E15.5 embryos because all the double-mutant embryos died by E17.5. Explant cultures mimic the in vivo retinal development well (Tomita et al., 1996a). After 2 weeks of culture (day 14), bipolar cells were differentiated in the wild-type retinal explants, whereas in the double mutants bipolar cells were completely missing and instead Müller glial cells (GS positive, cyclin D3 positive) were increased (Fig. 1B,E,H,K), as previously described (Tomita et al., 2000). In the wild-type retina, Chx10 was expressed at a high level in the outer and inner regions of the INL but at a very low level in the middle region of the INL, where Müller glial cells are present (Fig. 1A,G). In contrast, in Mash1-Math3 double-mutant retina, Chx10 expression was observed more uniformly in the INL (Fig. 1D,J) and remained in Müller glial cells (Fig. 1D,F,J-L). In the double-mutants, about 50% of the Chx10-positive cells expressed GS and cyclin D3, whereas in the wild type virtually none of the Chx10-positive cells did (Fig. 1M). Thus, Chx10 expression per se is compatible with glial development and not sufficient for specification of the bipolar cell identity. Furthermore, these results indicate that the defects of loss of bipolar cells and concomitant increase of Müller glial cells in Mash1-Math3 double-mutant retina were not the results of a loss of Chx10 expression.

**Misexpression of bHLH genes promotes generation of rods but not of bipolar cells**

To further define the role of bHLH and homeobox genes in bipolar cell development, each gene was misexpressed with retrovirus in the developing retina. We used a replication-incompetent retrovirus, CLIG, which directs expression of...
green fluorescent protein (GFP) as a marker from the upstream LTR promoter (Fig. 2A) (Hojo et al., 2000). A test gene, such as one of the bHLH and homeobox genes, was inserted into the upstream of the internal ribosomal entry site (IRES) so that both the test and GFP genes were expressed bicistronically (Fig. 2A). Retinal explants were prepared from mouse embryos and infected with CLIG (B), CLIG-Mash1 (C), CLIG-Math3 (D) and CLIG-Ngn2 (E). After two weeks, the explants were subjected to immunohistochemistry using anti-GFP antibody. (F-I) Ratios of retinal cell types infected with CLIG (F), CLIG-Mash1 (G), CLIG-Math3 (H) and CLIG-Ngn2 (I). Rod genesis is increased by misexpression of Mash1, Math3 and Ngn2. (J) Neuronal ratios of the virus-infected cells. Almost all of the cells infected with CLIG-Mash1, CLIG-Math3 and CLIG-Ngn2 became neurons. (K) Gliogenic ratios of the virus-infected cells. Gliogenesis was suppressed by misexpression of Mash1, Math3 and Ngn2. Each bar is the average of at least two independent samples. Scale bar: 25 μm.

Fig. 3. Misexpression of homeobox genes. (A) Schematic structure of retroviral vectors. Three repeats of the Myc epitope were fused at the N terminus of Chx10 and Pax6. (B-I) Retinal explants were prepared from E17.5 mouse embryos and infected with CLIG-Chx10 (B,D,F,H) and CLIG-Pax6 (C,E,G,I). After 2 weeks, the explants were subjected to immunohistochemistry using anti-Myc, anti-GFP, anti-PKC, anti-GS and anti-cyclin D3 antibodies, as indicated in each figure. The majority of the virus-infected cells were located in the INL. As GFP staining was very weak in the cells infected with these viruses, both Myc and GFP expression was detected by the same fluorescence (B,C). Few cells infected with CLIG-Chx10 or CLIG-Pax6 expressed the bipolar cell marker PKC (D,E) while some of the CLIG-Chx10-infected cells expressed the Müller glial markers GS (F, arrows) and cyclin D3 (H, arrows). (J,K) Ratios of cell numbers in the GCL, INL and ONL. Misexpression of Chx10 (J) and Pax6 (K) significantly increased the population of the INL cells. Each bar is the average of at least four independent samples. Scale bar: 25 μm.

mostly differentiated into bipolar and Müller glial cells in the INL (Fig. 2B,F), as previously described (Turner and Cepko, 1987). In contrast, when CLIG-Mash1 or CLIG-Math3 was applied, almost all virus-infected cells became rods, the most ‘preferred’ cell fate during this culture period (Fig. 2C,D,G,H). However, Mash1 or Math3 alone did not promote bipolar cell genesis. This activity is similar to that of NeuroD, which promotes rod genesis (Morrow et al., 1999). We also examined another bHLH gene, Ngn2, which is expressed by retinal precursor cells but downregulated during cell differentiation
Cell type specification by homeobox and bHLH genes

(Gradwohl et al., 1996; Sommer et al., 1996). Misexpression of \textit{Ngn2} also promoted rod differentiation, although less efficiently (Fig. 2E,I). Thus, although these bHLH genes alone cannot determine the correct neuronal subtypes, they all promote generation of neurons, mostly rods (Fig. 2J).

Interestingly, misexpression of these bHLH genes almost completely inhibited gliogenesis (Fig. 2K), as previously described (Brown et al., 1998; Morrow et al., 1999; Bae et al., 2000; Cai et al., 2000).

**Misexpression of homeobox genes promotes generation of INL cells but not of mature bipolar cells**

To determine the function of homeobox genes, \textit{Chx10} and \textit{Pax6} were misexpressed in the retinal explants. Each gene with or without three repeats of the Myc epitope (Fig. 3A) was misexpressed and the same results were obtained. Both \textit{Chx10} and \textit{Pax6} are expressed in the INL, but the former is specifically expressed by bipolar cells (Liu et al., 1994) while the latter is expressed by amacrine cells (Davis and Reed, 1996; Xu et al., 1999). When CLIG-Chx10 was applied, nearly 90% of the virus-infected cells were located in the INL and only 10% became rods in the ONL (Fig. 3B). Thus, \textit{Chx10} dramatically increased the population of the INL cells. Approximately 65% of the CLIG-Chx10-infected cells were found in the middle region of the INL, where bipolar and Müller glial cells are located. As 25% of them had a Müller glial morphology, 40% could be bipolar cells (Fig. 3B), in contrast to the CLIG infection, where only 6% became bipolar cells (Fig. 2F). However, these \textit{Chx10}-induced bipolar-like cells were mostly negative for the neuronal marker NSE (see Fig. 7A) and the bipolar-specific marker PKC (Fig. 3D), suggesting that they were still immature. To accurately count the PKC-positive cells, the retinal explants infected with virus were dissociated and subjected to immunocytochemistry. Only 5.74±0.99% of the CLIG-Chx10-infected cells were positive for PKC, whereas 3.27±0.45% of the CLIG-infected cells were PKC positive (Table 1). Thus, although \textit{Chx10} exhibited a more than sixfold increase (40% versus 6%) in the number of bipolar-like cells, it generated only 1.8-fold more (5.74±0.99% versus 3.27±0.45%) of PKC-positive mature bipolar cells.

In addition to bipolar-like cells, 15% of the CLIG-Chx10-infected cells were located at the outer regions of the INL and 10% of them at the inner regions of the INL, which contain horizontal and amacrine cells, respectively. These results suggest that a total of 25% of the CLIG-Chx10-infected cells became horizontal- and amacrine-like cells. However, immunocytochemical analysis of dissociated retinal cells indicated that only 6.54±0.71% of the CLIG-Chx10-infected cells expressed the horizontal and amacrine cell-specific marker calbindin, although this ratio was significantly higher than that of the CLIG-infected cells (Table 1). These results suggest that, although \textit{Chx10} alone can induce generation of the INL cells, many of them did not become mature.

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**Fig. 4.** Prolonged retinal explant cultures misexpressing homeobox genes. Retinal explants were prepared from E17.5 mouse embryos and infected with CLIG-Chx10 (A-F) and CLIG-Pax6 (G-L), and the fates of the virus-infected cells were examined at day 18 of culture. (A-F) Even at day 18, the majority of CLIG-Chx10-infected cells were negative for PKC (A-C) and NSE (D-F). (G-L) At day 18 of culture, the majority of CLIG-Pax6-infected cells were negative for PKC (G-I) and NSE (J-L). Scale bar: 25 μm.

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**Table 1. Ratios of virus-infected cells**

<table>
<thead>
<tr>
<th>Infected virus</th>
<th>PKC positive</th>
<th>GS positive</th>
<th>Cal positive</th>
<th>Rhodopsin positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLIG (n=5)</td>
<td>3.27±0.45% (28/857)</td>
<td>8.44±2.11% (76/900)</td>
<td>0.81±0.33% (10/1236)</td>
<td>82.84±0.71% (676/816)</td>
</tr>
<tr>
<td>CLIG-Chx10 (n=6)</td>
<td>5.74±0.99% (27/470)</td>
<td>33.11±1.89% (145/438)</td>
<td>6.54±0.71% (28/428)</td>
<td>16.8±0.41% (76/452)</td>
</tr>
<tr>
<td>CLIG-Chx10-Mash1 (n=8)</td>
<td>11.21±0.85% (269/2399)</td>
<td>22.55±0.96% (475/2106)</td>
<td>8.10±0.57% (217/2663)</td>
<td>10.88±1.33% (120/1103)</td>
</tr>
<tr>
<td>CLIG-Chx10-Math3 (n=5)</td>
<td>26.26±3.06% (240/914)</td>
<td>9.45±1.56% (76/804)</td>
<td>5.37±1.44% (38/708)</td>
<td>13.21±0.77% (104/787)</td>
</tr>
<tr>
<td>CLIG-Chx10-Hes5 (n=3)</td>
<td>6.70±0.70% (30/448)</td>
<td>53.65±2.08% (176/328)</td>
<td>6.21±0.53% (27/435)</td>
<td>12.1±3.63% (43/354)</td>
</tr>
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</table>

After two weeks of culture, retinal explants infected with retrovirus were dissociated and subjected to immunocytochemistry. The ratios with s.e.m. (%) of marker positive cells (marker positive cells/total infected cells) were determined. The number of independent explant cultures is indicated (n).
interneurons. In addition to these neuron-like cells, Chx10 induced generation of Müller glia, which expressed glutamine synthetase (GS) (Fig. 3F, arrows) and cyclin D3 (Fig. 3H, arrows). Surprisingly, 33.11±1.89% of the CLIG-Chx10-infected cells were positive for GS (Table 1). These results suggest that Chx10 per se has a potential to generate all the cell types in the INL and cannot determine the neuronal versus glial fate specification, agreeing with the observation that Chx10 is expressed by Müller glial cells in Mash1-Math3 double mutants.

Interestingly, misexpression of Pax6 also promoted generation of the INL cells and inhibited the ONL cell development (Fig. 3C,K). However, these INL cells were morphologically immature and did not express neuronal markers such as PKC (Fig. 3E) and calbindin (data not shown) or the glial markers GS (Fig. 3G) and cyclin D3 (Fig. 3I). These results suggest that the homeobox genes confer the INL-specific identity but are not sufficient for generation of mature neurons.

To examine the possibility that neuronal differentiation of the virus-infected cells is only delayed by misexpression of Chx10 and Pax6, the retinal explants were cultured for 18 days. However, even at this stage, the majority of the CLIG-Chx10- and CLIG-Pax6-infected cells were negative for PKC (Fig. 4A-C,G-I) and NSE (Fig. 4D-F,J-L), thus excluding the possibility that neuronal differentiation is only delayed.

Since the INL neuron-like cells induced by Chx10 and Pax6 were negative for PKC and NSE, it is possible that they could be still precursor cells. To examine this possibility, the precursor-specific markers cyclin D1 and nestin were examined. However, the INL neuron-like cells induced by Chx10 or Pax6 alone were all negative for such markers (data not shown, see Fig. 7I), suggesting that they were not precursors. These results indicate that, although the homeobox genes direct precursors to become the INL cells, including
Müller glia, they are not sufficient for generation of mature interneurons.

Co-expression of bHLH and homeobox genes promotes generation of mature bipolar cells

We next examined the effects of co-expression of bHLH and homeobox genes. For co-expression of the two genes, the bHLH genes were fused with GFP and each of them was co-expressed with Chx10 (Fig. 5A). More than 99% of the cells infected with these retroviruses successfully co-expressed both bHLH and homeobox genes (Myc positive and GFP positive) (data not shown). The cell types were determined immunochemically after sectioning or dissociating cultured retinal explants. Co-expression of Chx10 and Mash1 moderately increased the population of PKC-positive bipolar cells (Fig. 5C,E, arrows; Fig. 6A), compared with misexpression of Chx10 alone (11.21% versus 5.74%; Table 1). In addition, this co-expression moderately decreased the GS-positive Müller glial cell population (Figs 5D,F, 6B; Table 1). Co-expression of Chx10 and Math3 more significantly increased the population of PKC-positive bipolar cells (Figs 5H,J, 6A), compared with misexpression of Chx10 alone (26.26% versus 5.74%, Table 1). In addition, this co-expression significantly decreased the GS-positive Müller glial cell population (9.45% versus 33.11%) (Figs 5I,K,6B and Table 1). Thus, Mash1 and Math3 promoted bipolar cell development, when Chx10 was also expressed, but did not induce rod genesis (Fig. 6D) although they alone significantly increased the rod population, indicating that the activities of Mash1 and Math3 are context dependent. Interestingly, co-expression of Chx10 and Mash1 or Math3 did not significantly affect generation of calbindin (Cal)-positive cells (horizontal and amacrine cells) (Fig. 6C), suggesting that a combination of Chx10 and Mash1 or Math3 may be more specific for bipolar cell genesis.

In contrast, co-expression of Chx10 and Hes5, a bHLH repressor known to antagonize Mash1 and Math3 (Akazawa et al., 1992), dramatically increased generation of Müller glial cells (Figs 5N,P, 6B; Table 1). These results suggest that the activity of Chx10 for bipolar cell genesis is also context dependent and that, while Chx10 induces INL-specific cell types, it alone has a potential to promote development of both bipolar and Müller glial cells.

To further characterize the cells induced by Chx10 and Mash1 or Math3, we used other markers for INL cells. In the INL, NSE is specifically expressed by mature bipolar cells while cyclin D3 is expressed by Müller glia. Although few CLIG-Chx10-infected cells were NSE positive (Fig. 7A), co-expression of Chx10 with Mash1 or Math3 significantly increased the population of NSE-positive bipolar cells (Fig. 7B,C, arrows). In addition, co-expression of Chx10 with Mash1 or Math3 significantly decreased the population of cyclin D3-positive Müller glial cells (Fig. 7E-G). In contrast, co-expression of Chx10 and Hes5 decreased NSE-positive bipolar cells (Fig. 7D), while increasing cyclin D3-positive Müller glial cells (Fig. 7H, arrows). The precursor-specific marker cyclin D1 was negative in all the virus-infected cells (Fig. 7I-L). These data indicate that, whereas Chx10 alone generates mostly immature neurons or Müller glial cells, co-expression of Chx10 and Mash1 or Math3 efficiently induces bipolar cell fate specification.

Cell proliferation and death are not induced by bHLH and homeobox genes

The increase of bipolar cells by Chx10 and Mash1 or Math3 could be the result of proliferation of bipolar cells and apoptosis of other cell types rather than conversion of precursors to the bipolar cell fate at the expense of other cell
types. To distinguish between these possibilities, proliferation and death of virus-infected cells were analyzed at days 3, 4, 7 and 14 of the cultures. Cell proliferation was examined using Ki67, a nuclear antigen expressed by proliferating cells. The majority of the cells infected with CLIG, CLIG-Chx10-Mash1, or CLIG-Chx10-Math3 were negative for Ki67 at all the time points (Fig. 8A–F; data not shown), indicating that Chx10 and Mash1 or Math3 did not promote cell proliferation. To determine the extent of cell death, the retinal explants were subjected to TUNEL assay. The majority of the virus-infected cells were negative for the TUNEL assay at all the time points (Fig. 8G–L; data not shown). These results suggest that the bipolar cell genesis induced by Chx10 and Mash1 or Math3 was not the result of proliferation of bipolar cells or apoptosis of other cell types but most likely of conversion of precursors toward the bipolar cell fate at the expense of other cell types.

To further determine the extent of cell proliferation and survival, the clonal size of the virus-infected cells was examined. The sizes of clones infected with CLIG-Chx10-Mash1 and CLIG-Chx10-Math3 were mostly one or two cells, and they were very close to the size of CLIG-infected clones (Fig. 8M,N). These results suggest that Chx10 and Mash1 or Math3 did not affect cell proliferation or death, agreeing with the above data of Ki67 staining and TUNEL assay.

**DISCUSSION**

A combination of Chx10 and Mash1 or Math3 induces bipolar cell genesis

Previous studies revealed that the bHLH genes Mash1 and Math3 and the homeobox gene Chx10 are required for bipolar cell genesis, but their exact functions remained to be determined. We have found that the complete loss of bipolar cells by Mash1-Math3 double mutation does not involve Chx10, indicating that the bHLH and homeobox genes have distinct functions in bipolar cell genesis. We further examined the functions of the bHLH and homeobox genes by misexpressing them in the developing retina. Misexpression of the bHLH genes alone promoted generation of rods, the most ‘preferred’ cell type, but did not induce bipolar cell genesis. All the bHLH genes that we tested (Mash1, Math3 and Ngn2) promoted rod genesis, although they are not expressed by rods. Thus, we did not observe any specific activities of these bHLH genes in the generation of the correct cell types. In contrast, misexpression of Chx10 promoted generation of INL cells, but most of them were still immature interneurons or Müller glial cells. Thus, Chx10 is not sufficient either to generate mature bipolar cells. Chx10 may endow the INL-specific identity but not the neuronal lineage information. Strikingly, co-expression of Mash1 or Math3 together with Chx10 significantly promoted bipolar cell genesis. Thus, the activity of the bHLH genes is context-dependent and, when Chx10 is expressed, Mash1 and Math3 did not promote rod genesis but promoted bipolar cell genesis. In addition, these bHLH genes inhibited generation of Müller glial cells, indicating that the main function of bHLH genes is the neuronal versus glial fate determination. Thus, homeobox and bHLH genes confer the different information on cells: Chx10 gives the layer-specific identity while Mash1 and Math3 give the neuronal lineage.

![Fig. 8. Cell proliferation and death. (A–F) Retinal explants infected with CLIG (A,D), CLIG-Chx10-Mash1 (B,E) and CLIG-Chx10-Math3 (C,F) were subjected to immunohistochemistry with anti-Ki67 antibody (red) at days 3 (A–C) and 7 (D–F) of cultures. The majority of the virus-infected cells were negative for Ki67 expression. (G–L) Retinal explants infected with CLIG (G,J), CLIG-Chx10-Mash1 (H,K) and CLIG-Chx10-Math3 (I,L) were subjected to TUNEL assay at days 3 (G–I) and 7 (J–L) of cultures. The majority of the virus-infected cells were negative for TUNEL assay. Scale bar: 25μm. (M) Comparison of the sizes of CLIG- and CLIG-Chx10-Mash1-infected clones. (N) Comparison of the sizes of CLIG- and CLIG-Chx10-Math3-infected clones. The clonal sizes are very similar in all infections, indicating that misexpression of Chx10 and Mash1 or Math3 did not affect cell proliferation or survival. For clonal size analysis, ten times diluted virus solution compared with A–L was used to clearly identify clones.](image-url)
information. Together these genes specify the bipolar cell fate in the retina.

Interestingly, whereas Chx10 also generated immature horizontal- and amacrine-like cells, co-expression of Mash1 or Math3 with Chx10 did not increase the population of mature horizontal or amacrine cells. These results suggest that the bHLH genes may not simply promote all types of neurogenesis but be involved in specification of subtype identities. The notion that bHLH genes are involved in specification of neuronal subtypes is supported by gene swapping experiments of both invertebrates and vertebrates. In Drosophila, the bHLH genes scute and atonal promote external sensory and chordotonal organs, respectively, and atonal mutation is not rescued by scute, even though the latter is forced to express in the chordotonal lineage cells (Chien et al., 1996). Domain swapping experiments indicate that the basic region is responsible for the specificity of the neuronal subtypes (Chien et al., 1996). Similarly in mammals, knock-in of Mash1 gene into the Ngn2 locus demonstrates that Mash1 partially rescues Ngn2 mutation but confers a different identity on rescued neurons (Fode et al., 2000). Our results indicate that Mash1 or Math3 may be also involved in neuronal subtype specification in addition to neuronal versus glial fate determination: these bHLH genes may endow the bipolar cell identities but not horizontal or amacrine cell identities. Our data also suggest that Mash1 or Math3 can specify the related neuronal subtype only within the correct layer, which is determined by Chx10, whereas outside of the layer they merely induce an unrelated neuronal subtype.

Although the homeobox and bHLH factors are important for bipolar cell genesis, it is still not clear how these two classes of transcription factors cooperate inside the cell. It is possible that they could synergistically upregulate the promoter activity of bipolar cell-specific genes. Specific interaction between homeobox and bHLH factors have been recently reported for the pituitary pro-opiomelanocortin (Pomc) gene (Poulin et al., 2000). The homeobox factor Pitx1 and the bHLH factor Pan1 physically interact with each other through the homeobox and bHLH domains and this interaction synergistically upregulates the Pomc promoter activity (Poulin et al., 2000). Thus, it is possible that Chx10 and Mash1 or Math3 interact with each other and synergistically promote expression of bipolar cell-specific genes such as the gene for PKC.

Other combinations of bHLH and homeobox genes may generate different cell types

It has been shown that the homeobox gene Crx and the bHLH gene NeuroD are required for photoreceptor development (Furukawa et al., 1999; Morrow et al., 1999), suggesting that a combination of these homeobox and bHLH genes may specify the photoreceptor identity. Similarly, a combination of other homeobox and bHLH genes may be involved in specification of horizontal and amacrine cell fates. Although a previous study indicated that Pax6, which is expressed by amacrine cells, does not have any clear activity in Xenopus retina (Hirsch and Harris, 1997), we demonstrated that Pax6 can promote generation of INL cells in mouse retina. This discrepancy could be due to different conditions for misexpression: the study with Xenopus shows transient misexpression whereas ours is permanent. Although Pax6 induced only immature INL cells, it could specifically promote amacrine cell genesis when an appropriate bHLH gene is also co-expressed. It remains to be determined which bHLH genes promote amacrine cell genesis with Pax6. The bHLH gene NeuroD is shown to increase the amacrine cell population (Morrow et al., 1999) and thus it is one of the candidates to specify the amacrine cell fate with Pax6. Various combinations of homeobox and bHLH genes should be further tested to elucidate the transcriptional code for the neuronal subtype identity.

Development of bipolar and Müller glial cells

Strikingly, Chx10 alone can induce many Müller glial cells and a combination of Chx10 and Hes5 generates even more abundant Müller glial cells, suggesting that Chx10 may be neutral to the neuronal and glial fate specification. In the absence of Mash1 and Math3, bipolar cells are completely missing and Müller glial cells are increased, although Chx10 expression is maintained. As this defect occurs without cell proliferation or death (Tomita et al., 2000), it is likely that the cells (Chx10 positive) that normally differentiate as bipolar cells adopt the glial fate in Mash1-Math3 double-mutant retina. Thus, Chx10-positive cells seem to differentiate into bipolar cells when Mash1 or Math3 is expressed, while differentiating into Müller glia when neither are expressed. Hes5, which is known to functionally antagonize the neuronal bHLH genes such as Mash1 (Akazawa et al., 1992), may produce the same effects as the Mash1-Math3 double mutation, thereby promoting gliogenesis. Similar to Hes5 (Hojo et al., 2000), it has been shown that misexpression of Hes1 and an active form of Notch also promotes Müller glial development (Furukawa et al., 2000). Because both Hes1 and Hes5 are Notch effectors (Kageyama and Nakanishi, 1997; Ohtsuka et al., 1999), these data indicate that activation of Notch signaling induces glial fate determination at the expense of bipolar cells. Because bipolar and Müller glial cells differentiate from common precursors, these results raise an interesting hypothesis that the progeny of the common precursors adopt the Müller glial cell fate when Notch signaling is activated, while adopting the bipolar cell fate when Notch signaling is not activated. If this is the case, one of the progeny, which adopts the bipolar cell fate, might express a Notch ligand and activate the Notch pathway of its neighboring progeny, which would become Müller glial cells. Gliogenic activity of Notch signaling is also reported in the telencephalon and neural crest cells (Gaiano et al., 2000; Morrison et al., 2000). Further study of Notch signaling should help us to understand the mechanism for binary cell fate specification between bipolar and Müller glial cells.

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