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Math5 is required for both early retinal neuron differentiation and cell cycle progression

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Abstract

CNS progenitors choose a fate, exit mitosis and differentiate. Basic helix–loop–helix (bHLH) transcription factors are key regulators of neurogenesis, but their molecular mechanisms remain unclear. In the mouse retina, removal of the bHLH factor *Math5* (*Atoh7*) causes the loss of retinal ganglion cells (RGCs) and appearance of excess cone photoreceptors. Here, we show a simultaneous requirement for *Math5* in retinal neuron formation and cell cycle progression. At embryonic day E11.5, *Math5−/−* cells are unable to assume the earliest fates, particularly that of an RGC, and instead adopt the last fate as Müller glia. Concurrently, the loss of *Math5* causes mitotically active retinal progenitors to undergo aberrant cell cycles. The drastic fate shift of *Math5−/−* cells correlates with age-specific alterations in *p27/Kip1* expression and an inability to become fully postmitotic. Finally, *Math5* normally suppresses *NeuroD1* within *Math5*-expressing cells and inhibits *Ngn2* expression and cone photoreceptor genesis within separate cell populations. Thus, *Math5* orchestrates neurogenesis in multiple ways, regulating both intrinsic and extrinsic processes.

Keywords: Retina; RGC; Mouse; bHLH; *Math5*; *Ngn2*; *NeuroD1*; Neurogenesis; Cell cycle progression

Introduction

In the forming nervous system, neuroepithelial cells are multipotent but ultimately choose unique fates. The vertebrate retina is a good model for investigating the mechanisms of fate determination since only seven basic neuronal and glial cell types arise. Neuronal birthdating in the rodent has demonstrated that retinal progenitor cells (RPCs) give rise to neurons or glia in an overlapping sequence (Carter-Dawson and LaVail, 1979; Rapaport et al., 2004; Sidman, 1961; Young, 1985a,b). In these studies, the timing of the terminal S phase or "birthdate" is closely correlated with retinal neuronal identity. Retinal ganglion cells (RGCs) are born first and Müller glia last in all vertebrates, with differing orders of appearance for the other classes. In mice, RGCs are immediately followed by horizontal, cone photoreceptor, and amacrine neurons. Later, rod photoreceptors arise, followed by bipolar neurons and Müller glia. Retinal birth order, lineage tracing (Holt et al., 1988; Turner et al., 1987, 1990; Wetts and Fraser, 1988), and heterochronic transplantation experiments (Austin et al., 1995; Wantanabe and Raff, 1990) all have contributed to the model of retinal progenitor competence states (Cepko et al., 1996). Here, RPCs integrate multiple inputs from intrinsic factors, cell–cell contact and secreted signals to acquire competence, producing different cell types across time (Livesey and Cepko, 2001).

Two transcription factor classes, basic helix–loop–helix (bHLH) and homeobox proteins, generate intrinsic cues that promote vertebrate retinal neurogenesis. This was demonstrated by loss- and gain-of-function experiments that alter fates within

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specific retinal neuron populations (reviewed in Hatakeyama and Kageyama, 2004; Livesey and Cepko, 2001; Vetter and Brown, 2001). There are five known retinal neuron-promoting bHLH genes: *Ath5*, *Ngn2*, *Ath3*, *NeuroD1*, and *Ash1*. In the mouse retina, transcription of each gene is activated in sequence, across several days of development (Brown et al., 1998), and coincides with the peak of genesis for particular retinal cell type(s). For example, mouse *Ath5* (*Atoh7* or *Math5*) initiates first, followed by *Ngn2* and then *NeuroD1*.

In the mammalian eye, *Math5* is critically required for retinal ganglion cell (RGC) genesis (Brown et al., 2001; Wang et al., 2001). Loss- and gain-of-function studies in frog, zebrafish, and chick have also defined a key role for *Ath5* in RGC formation (Kanekar et al., 1997; Kay et al., 2001; Liu et al., 2001). *Math5* expression begins at E11 in the dorso-central optic cup but disappears from the retina at early postnatal ages (Brown et al., 1998). Targeted deletion of *Math5* causes a profound loss of RGCs (Brown et al., 2001; Wang et al., 2001). In each report, mutant retinal cells that normally form RGCs were proposed to switch their primary fate from the first neuron class to one that closely follows; however, the mechanisms for this change have not been further explored. Recent retinal lineage-tracing experiments show that *Math5*-expressing cells exhibit broad developmental potential (Brzezinski, 2005; Yang et al., 2003) but are neither proliferating progenitors nor differentiated neurons. This suggests that *Math5*-expressing cells represent the earliest retinal competence group, with *Math5* function necessary but insufficient for RGC fate. In addition, *Math5*-expressing cells can be described as "transitional" (Dyer and Bremner, 2005), since they are nonmitotic, migratory cells that are undifferentiated but committed to particular fates. Interestingly, the transitional classification of *Ath5* cells appears evolutionarily conserved since *Drosophila atonal* is found in G_1-arrested retinal cells and chick and zebrafish *Ath5* are excluded from S phase RPCs (Jarman et al., 1994; Jarman et al., 1995; Ma et al., 2004; Masai et al., 2005).

In this paper, we report the in vivo consequences of *Math5* loss on both embryonic retinal fate determination and the progenitor cell cycle. *Math5*−/− cells are unable to adopt the earliest fates and instead differentiate as Müller glia, the last fate. This drastic shift is associated with fluctuations in the expression of the CDK inhibitor *p27/Kip1* within those retinal cells that normally express *Math5*. In addition, *Math5* is required for surrounding RPC cell cycle progression and cone photoreceptor differentiation. Finally, we demonstrate that Math5 normally inhibits two bHLH genes, *NeuroD1* and *Ngn2*, which promote photoreceptor fates, directly or indirectly. Thus, *Math5* simultaneously influences the competence of different retinal cell populations in part by regulating other proneural genes.

Experimental procedures

Animals

Math5 (*Atoh7*) targeted deletion mice are described in Brown et al. (2001). This mutation is maintained in several genetic backgrounds including CD-1 and 129S1/SvImJ used in these studies. For embryonic staging, the day a copulation plug was observed was designated E0.5. Throughout this paper, *Math5*^{lacZki} refers to βgal expression from a *lacZ* gene homologously recombined into the *Math5* locus, while *Math5*+/− and *Math5*−/− indicate heterozygous or homozygous loss of *Math5*.

Pregnant mice carrying litters between the ages of E11.5 and E15.5 were injected once intraperatonially with bromodeoxyuridine (BrdU) at 0.1 mg/g body weight of BrdU/0.9M NaCl. For birthdating experiments, eyes were collected at P1 or P21, fixed for 1 h or overnight in 4% paraformaldehyde (PFA)/PBS, OCT embedded, cryosectioned, and antibody labeled (see next section). In pulse labeling studies, embryos were collected 1.5 h postinjection, fixed in 4% PFA/PBS for 1 h, and processed for cryosectioning and antibody labeling. Genotypes were determined by PCR (Brown et al., 2001). BrdU-labeled sections were treated with 2N HCl/0.5%Triton/PBS for 60 min prior to antibody blocking. Because retinal neurogenesis is influenced by the pigmented RPE (Rachel et al., 2002), birthdating experiments were performed in both albino (CD-1) and agouti ($\geq N_7F_2$ 129) backgrounds. No differences in the timing, number or type of retinal neuron population shifts were found between albino and pigmented retinal eyes.

Immunohistochemistry

Sections of embryonic or adult retinal tissue were processed for antibody labeling as previously reported (Mastick and Andrews, 2001). Antibodies used were rabbit anti-βIII-tubulin (1:1000 (TuJ1) Covance), mouse anti-Syntaxin (1:500, Sigma), rabbit anti-Calretinin (1:2000, Chemicon), sheep anti-Chx10 (1:2000, Exalpha N-terminal antibody), mouse anti-NF160 (1:50, Sigma), goat anti-Brn3b (1:100, Santa Cruz), rabbit anti-RXRγ (1:250, Santa Cruz), rat anti-βgal (1:500, a gift from Sean Carroll), rabbit anti-βgal (1:5000, ICN), mouse anti-p27/Kip1 (1:100, clone 57, BD/Invitrogen), rabbit anti-Ki67 (1:1000, Vector Labs), mouse anti-cyclin D1 (1:500, Santa Cruz), rabbit anti-phosphorylated histone H3 (1:200, Upstate Biotechnology), rabbit anti-Ngn2 (1:1000, a gift from Masato Nakafuku), rabbit anti-NeuroD1 (1:100, Santa Cruz, N-19 peptide antibody), rat anti-BrdU (1:100, Serotec), mouse anti-BrdU (1:100, Becton Dickinson), and mouse anti-CRALBP (1:2000, a gift from Tom Glaser and John Saari). Directly conjugated or biotinylated secondary and streptavidin-conjugated tertiary antibodies were obtained from Jackson ImmunoResearch Laboratories, Molecular Probes or Vector Labs.

TUNEL

Embryonic sections were fixed for 20 min with 4% PFA/PBS, processed using manufacturer's instructions in the In Situ Cell Death Detection Kit, TMR red (Roche) and counterstained with DAPI.

Cell counting

Birthdating

Wild type and *Math5*−/− eyes from three or more independent litters were birthdated once at E11.5 to E11.5 and analyzed at P1 or P21. For the P21 analysis, one 129 and two CD-1 litters were analyzed at each age. Retinal sections were judged at equivalent depth through each eye by selecting matched serial sections between wild type and mutant eyeballs. For each age and genotype, two non-adjacent 10-μm sections were analyzed. Three adjacent fields within the central retina were quantified from each section. Nuclei completely filled with BrdU labeling were quantified in each retinal cell layer (GCL, INL, ONL) and normalized against DAPI-labeled cells using Openlab (Improvision) counting software. Statistically significant differences were determined by a Student's *t* test.

Embryonic retinal cells

Wild type and *Math5*−/− embryonic heads were collected from three independent CD-1 litters at each age, E11.5–E15.5. Equivalent retinal sections were selected for comparison from a series, using anatomical landmarks in the embryonic head. BrdU-labeled and DAPI-labeled nuclei throughout the entire optic cup were tabulated. Two independent sections (separated by several sections) for each genotype and age were analyzed and significant differences determined by Student's *t* test.

The total number of Brn3b+, Brn3b+BrdU+, Ngn2+, Ngn2+BrdU+, NeuroD1+, NeuroD1+βgal+ or Ngn2+βgal+ retinal cells were quantified per 200× or 400× microscopic field. The average number of p27/Kip1+ or Phosphohistone H3+ (PhosHis+) cells was determined per embryonic retinal