RESEARCH COMMUNICATION

Requirement for math5 in the development of retinal ganglion cells

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math5 is a murine orthologue of atonal, a bHLH proneural gene essential for the formation of photoreceptors and chordotonal organs in Drosophila. The expression of math5 coincides with the onset of retinal ganglion cell (RGC) differentiation. Targeted deletion of math5 blocks the initial differentiation of 80% of RGCs and results in an increase in differentiated amacrine cells. Furthermore, the absence of math5 abolishes the retinal expression of brn-3b and the formation of virtually all brn-3b-expressing RGCs. These results imply that math5 is a proneural gene essential for RGC differentiation and that math5 acts upstream to activate brn-3b-dependent differentiation processes in RGCs.

Received September 27, 2000; revised version accepted November 20, 2000.

The mammalian retina is the peripheral portion of the visual system containing six major neuronal cell types and one glial cell type organized in a laminar structure. The visual information process in the retina follows a general pathway from photoreceptors to bipolar cells to retinal ganglion cells (RGCs). Horizontal cells and amacrine cells act to mediate lateral interactions among photoreceptors, bipolar cells, and RGCs. The latter serve as the sole output neurons in the retina to send the visual information down the optic nerve to the rest of brain. Both birthdating experiments using 3H-thymidine labeling and cell lineage analysis using retroviral and tracer-mediated approaches demonstrate that vertebrate retinal neurons are generated from common progenitors through sequential differentiation and ordered migration to form the laminar retinal structure (Cepko et al. 1996). Current models for retinal neuron differentiation suggest that the formation of a specific retinal neuron is determined by the intrinsic properties of the retinal progenitor and the extrinsic cues from the retinal environment (Cepko et al. 1996). Among the likely intrinsic factors, the basic helix–loop–helix (bHLH) class of proneural transcription factors appears to play an essential role in regulating the differentiation of retinal neurons (Cepko 1999). In Drosophila, expression of proneural genes of the achaete-scute complex (AS-C) and atonal (ato) in proneural clusters endow cells with neural competence (Jan and Jan 1993). Loss-of-function mutation of genes in AS-C causes the cells in the would-be proneural clusters to adopt epidermal fates rather than neuronal precursor fates. Conversely, gain-of-function mutations in the proneural genes leads to the ectopic formation of sensory neurons (Campuzano and Modolell 1992). The expression of ato is found in the optic furrow of the eye-antennal disc in addition to the ectodermal proneural clusters and sensory organ precursors, which give rise to the chordotonal organs. Deletion of ato causes the absence of the chordotonal organ and the lack of photoreceptors (Jarman et al. 1995), and the reduced expression of ato results in defects in axonal pathfinding of photoreceptors (White and Jarman 2000), suggesting that ato plays dual roles in determining neuronal potential and regulating specific neuronal differentiation events. Murine orthologs of many Drosophila proneural genes, including NeuroD, mash1, math4a/Ngn2, math5, and math1, are expressed in the developing retina and are thought to function as positive regulators of neuronal differentiation programs (Brown et al. 1998; Cepko 1999). Muta-genes studies have shown that mash1 regulates the formation of late-born retinal neurons, particularly the bipolar cells, and NeuroD acts to control the differentiation of amacrine and bipolar cells (Tomita et al. 1996; Morrow et al. 1999). While the differentiation of RGCs, the first-born retinal neurons, begins at embryonic day 11.5 (E11.5; Young 1985a), the retinal expression of NeuroD, math1, and math4a/Ngn2 genes initiate at E13.5, and the retinal expression of mash1 is not detected until E14.5 (Brown et al. 1998), indicating that none of the above bHLH genes is responsible for the initial differentiation of RGCs.

math5, an ortholog of ato, is transiently expressed in developing mouse retinas starting at E11, and its expression coincides with the differentiation of RGCs (Brown et al. 1998). Thus, math5 is a good candidate for a proneural gene required for RGC formation. To investigate the role of math5 in mouse retinal development and, particularly, in the development of RGCs, we used homologous recombination in murine embryonic stem (ES) cells to delete math5. We show here that targeted deletion of math5 results in the loss of >80% of RGCs and of virtually all brn-3b-expressing RGCs and leads to an increase in differentiated amacrine cells. Expression properties of a reporter lacZ gene in math5-lacZ knock-in mice demonstrate that deletion of math5 does not prevent the formation of RGC progenitors. However, math5 deficiency leads to the loss of retinal expression of the
early RGC differentiation markers, brn-3b and p75, implying that the null mutation in math5 blocks the initial differentiation of RGCs. The results imply that math5 is essential for the initial differentiation of RGCs and that the loss of math5 is likely to cause a cell-fate conversion of retinal progenitors from RGCs to amacrine cells. The effect of the math5 mutation on the onset of brn-3b expression suggests that math5 acts upstream to activate brn-3b-dependent differentiation processes in RGCs.

Results and Discussion

Targeted deletions of math5 and retinal expression of a reporter lacZ gene

To investigate the role of math5 in vivo, we generated a targeted deletion of math5 by homologous recombination in E8 cells. The mutant math5 allele was created by replacing the entire math5 open reading frame (ORF) with either a lacZ or GFP reporter gene (Fig. 1). Mice heterozygous for the math5-null allele appeared normal with no apparent retinal defects. Both heterozygous and homozygous math5-mutant mice were viable and fertile and had no discernible physical defects.

Previous in situ hybridization studies have shown that the retinal expression of math5 was initiated at E11, before the onset of RGC differentiation. Its retinal expression was often observed in cells adjacent to proliferating regions (Brown et al. 1998). To determine whether the expression pattern of the knock-in lacZ gene faithfully reflected that of endogenous math5 and to evaluate the possible effects of the math5 mutation on retinal development, we compared math5-lacZ expression in the retinas of heterozygous and homologous mutant embryos. math5-lacZ expression in retinas of heterozygous embryos was first detected at E11 and peaked at E13.3, with math5-lacZ-expressing cells distributed uniformly from the ventricular [proliferating] zone to the vitreous side (retinal ganglion layer) of the retina [Fig. 2A,C,E]. After E13.5, lacZ expression was significantly reduced over the entire retina. At E15.5, robust expression was limited to the peripheral regions of the retina where RGCs were actively being generated, but much weaker expression was observed in more medial regions where RGCs were postmitatory (Fig. 2G). The lacZ expression in the ganglion cell layer (GCL) was likely to represent residual lacZ activity because endogenous math5 expression is not found in differentiated RGCs (Brown et al. 1998).

The overall expression pattern of lacZ in heterozygous embryonic retinas closely resembled that of endogenous math5 (Brown et al. 1998) and indicated that the math5 regulatory sequences in the math5-lacZ allele were sufficient to confer correct spatiotemporal expression. No notable differences in lacZ expression were seen when homozygous math5-lacZ/math5-GFP retinas from E11, E12.5, and E13.5 embryos were compared with similarly staged heterozygous math5-lacZ/+ retinas (Fig. 2A-F), suggesting that a comparable number of math5-expressing cells were generated in heterozygous and homozygous math5-mutant retinas and that the expression of math5 was not controlled by autoregulatory mechanisms as is the case for ato and math1 (Jarman et al. 1995, Helms et al. 2000). However, in math5-lacZ/math5-GFP retinas at E15.5, we found a complete absence of the residual lacZ expression in GCL, which contrasted with the readily observable albeit weak expression in math5-lacZ/+ retinas at the same stage [Fig. 2G,H]. These results indicated that very few differentiated RGCs were forming in math5-deficient retinas.

Retinal ganglion cell defects of math5-null mice

To assess the potential loss of RGCs in math5-null retinas, we analyzed retinal sections prepared from postnatal mice at 3 wk of age, a time when all retinal neurons are fully developed (Young 1985b). The overall laminar structure of the homozygous math5-deficient retinas resembled those of wild-type and heterozygous retinas, and no noticeable changes in the number of photoreceptor cells in the outer nuclear layer (ONL) were observed. However, the mutant retinas were ~15%–20% thinner [Fig. 3A,B]. This difference was mostly caused by the loss of >40% of the cells in GCL and the absence of a defined nerve fiber layer [NFL], which consists entirely of bundles of RGC axons [Fig. 3A,B]. In mice, RGCs and displaced amacrine cells each constitute about one-half of the cells in GCL (Perry 1981; Barnstable and Drager 1984). Therefore, the 40% or more loss of cells in GCL could account for a >80% loss of RGCs. Quantification of the number of axon bundles in retinas immunostained with SMI-32, a mouse monoclonal antibody that reacts with neurofilament H and labels predominantly the axons of large ganglion cells.
Figure 2. Expression of the math5-lacZ fusion gene in developing retinas. The math5 heterozygous [math5-lacZ/+] and homozygous [math5-lacZ/GFP] retinas were collected for whole-mount staining of lacZ activities. After staining, the retinas were cryosectioned and photographed using DIC-Nomarski optics. The math5-lacZ expression was first detected in the central region of developing retina at E11.5 [A,B]. At E12.5 (C,D) and E13.5 (E,F), math5-lacZ expression rapidly expanded throughout the retinas. After peaking at E13.5, high-level expression was limited to the peripheral regions of the retina (G,H), where RGCs are being generated. Scale bar, 100 µm.

[Wang et al. 2000]

Effects of math5 mutation on other retinal neurons

In Drosophila, ato is required for the specification of the founder photoreceptors— the R8 cells, which in turn recruit the other photoreceptors (Jarman et al. 1995). Current models of retinal neuron differentiation suggest that the cell differentiation of RGCs is negatively regulated through a feedback mechanism by the presence of differentiated RGCs (Cepko et al. 1996; Belliveau and Cepko 1999). However, it remains unclear how the loss of RGCs could influence the formation of other retinal neurons. In addition, the lack of appropriate synaptic connections in the absence of most RGCs is likely to have pronounced effects on neurons in the inner nuclear layer (INL). To determine whether the math5 mutation affected the development of retinal neurons other than RGCs, we monitored the expression of Protein Kinase C-α (PKC-α) as a bipolar cell marker (Wassle and Boycott 1991) and syntaxin and choline acetyltransferase (ChAT) as amacrine cell markers (Barnstable et al. 1985; Jeon et al. 1998) in mature retina.
PKC-α is expressed in cell bodies and processes of bipolar cells that mainly connect to rods [Wassle and Boycott 1991]. In wild-type retinas, PKC-α labeled cells in the inner nuclear layer and axons and dendrites within the outer and inner plexiform layer (Fig. 5A). In math5-null mutant retina, the labeling pattern and the number of PKC-α-expressing cells were similar to those in the wild-type retinas, indicating that math5-null mutation had no apparent effects on the bipolar cells expressing PKC-α.

In contrast to bipolar cells, amacrine cells were noticeably affected by the loss of math5. Expression of both syntaxin and ChAT were strongly upregulated when compared to wild-type controls (Fig. 5C–F). Quantitation of the number of ChAT-positive cells demonstrated a 7.5-fold increase in math5-null retinas (93 ± 12 ChAT-expressing cells/0.04 mm²) relative to wild-type controls (12.3 ± 3.3 ChAT-expressing cells/0.04 mm²). Moreover, most of the syntaxin- and ChAT-positive cells were located in GCL rather than the inner nuclear layer. In vertebrates, starburst amacrine cells are the only cholinergic retinal cells, and the cholinergic amacrine cells in GCL are the ON-starburst amacrine cells. Our data suggested that the loss of math5 had a significant effect on the amacrine cells in the GCL.
of \textit{math5} resulted in an increase of amacrine cells, in particular the ON-starburst amacrine cells, and their displacement to GCL. Most likely, the displacement of amacrine cells was caused by the loss of retinal ganglion cells. Based on cell birthdating experiments, amacrine cells are among the early-born neurons in the mouse retina and are generated at approximately the same time as RGCs [Cepko et al. 1996]. Thus, it is possible that in \textit{math5}-null retinas, progenitor cells unable to form RGCs switch their fate to the amacrine cell differentiation pathway. It is also possible that \textit{math5} is expressed in amacrine cell lineages during normal retinal development and negatively controls the differentiation of amacrine cells. However, if \textit{math5} acts like the other \textit{atonal}-class bHLH proneural genes to promote the differentiation of specific neurons, it is most likely that \textit{math5} is expressed only in RGC lineages and promotes their differentiation rather than in amacrine cell lineages to inhibit their differentiation.

Given that the majority of RGCs did not differentiate at early stages of retinal development, the fact that the resulting retina maintained its structural integrity and contained all other neuronal cell types was unexpected. Our results indicated that the developmental relationships among the retinal cell types are not tightly regulated and that the lack of majority of RGCs does not dramatically affect the overall patterning of the retina.

Several investigations have shown that the \textit{Notch-Delta} pathway is important for negatively regulating the differentiation of RGCs and other neurons in the retina [Austin et al. 1995; Dorsky et al. 1995; Bao and Cepko 1997]. The \textit{Notch-Delta} pathway likely acts to negatively regulate the expression of proneural genes like \textit{math5} in the mouse retina, as is the case for \textit{ato} in the \textit{Drosophila} eye. Because \textit{math5} encodes a bHLH transcriptional factor, it must function by regulating genes required for RGC differentiation. In \textit{Drosophila}, \textit{ato} functions to regulate the formation and axon pathfinding properties of R8 photoreceptors [White and Jarman 2000]. However, the molecular mechanisms of its function in axon pathfinding and other neural terminal differentiation processes remain poorly understood. Our studies showed that \textit{math5}- and \textit{brn-3b}-null mutations affect the development of largely the same population of RGCs and that \textit{math5} appears to be genetically upstream of \textit{brn-3b}. Combined with our early studies that \textit{brn-3b} is involved in normal axon growth of mouse RGCs [Gan et al. 1999, Wang et al. 2000], our results indicate that although the morphological and physiological properties of R8 photoreceptors in \textit{Drosophila} and RGCs in mice are highly diverged, a conserved genetic regulatory pathway may control the differentiation of both of these cell types. The down-regulation of the \textit{Notch-Delta} signaling pathway leads to the up-regulation of \textit{ato/math5}, which in turn activates downstream effector genes like \textit{brn-3b} to promote R8/RGC differentiation and normal axonal outgrowth. It remains to be determined whether \textit{math5} activates \textit{brn-3b} directly during normal retinal development. The results presented here demonstrate that the proneural gene \textit{math5} is essential for the differentiation of RGCs and that a null mutation of \textit{math5} results in the failure of the majority of RGCs to form.

Previous studies have shown that targeted expression of \textit{Xath5}, a \textit{math5} ortholog in \textit{Xenopus}, in the optic vesicle promotes the generation of RGCs, suggesting that \textit{Xath5} is sufficient to direct the differentiation of retinal progenitors into RGCs [Kanekar et al. 1997]. Whether or not \textit{math5} is sufficient for RGC in the mouse can be tested by ectopic expression of \textit{math5} in retinal progenitors. In addition, while \textit{ato} is required for the generation of all R8 cells, \textit{math5} was essential for the appearance of the majority of RGCs. It is likely that in mouse, other genes, particularly other proneural bHLH genes expressed in the retina, could account for the remaining RGCs.

\section*{Materials and methods}

\textbf{Targeted deletion of \textit{math5}}

Mouse \textit{math5} genomic sequences were isolated from a mouse genetic DNA library [Strain 129/SvE] in \textsc{ambah} [Stratagene], using \textit{math5} cDNA [L. Gan, unpubl.] as a probe. Targeted mutation of \textit{math5} was generated by inserting the \textit{math5} 1.5-kb 5' sequences containing the entire 5' untranslated region and the 5.8-kb 3' sequences into the \textsc{bamhi} (\textsc{xhoI} and \textsc{ecoRl}/\textsc{notI} sites in the 5' and 3' multiple cloning sites.
Retinal defects in math5 mutant mice

of pKI-lacZ or pKI-GFP vectors [L. Gan, unpubl.] respectively. The constructs place the lacZ and GFP under the control of math5 regulatory sequences. Generation of mutant mice, genotyping, and X-Gal staining were done as described [Gan et al. 1996, 1999]. All analysis was done on a mixed C57Bl/6J and 129/Sv/Ev background. To compare the number of brn-3b-lacZ-expressing cells, three math5 wild-type and three math5 mutant retinas from two litters at 3 wk old were whole-mount stained for lacZ activity, and the number of brn-3b-lacZ positive cells were counted in an 800 × 1200 µm field adjacent to the optic disc.

Sectioned in situ hybridization

Sectioned in situ hybridization was conducted as described [Riddle et al. 1993]. To generate brn-3b probes, the mouse 3.1-kb brn-3b cDNA clone was isolated from a mouse E14.5 retina cDNA library [L. Gan, unpubl.] in pBluescript SKII[+][Strategene]. The cDNA clone was linearized with Xhol and Xbal for synthesis of sense and antisense probes using T3 and T7 RNA polymerase, respectively.

Immunohistochemistry and microscopy

Immunohistochemical staining of whole-mount retinas using SMI-32 (Sternberger Monoclonals) was done as described [Gan et al. 1996]. Counts of SMI-32-expressing RGCs were compared in four wild-type and three math5 mutant retinas from mice at 3 wk old. To quantify the number of SMI-32-positive RGCs, micrographs of the whole-mount retinas were projected from eight optical sections with intervals of 0.5 µm. Projections were measured using MetaVue software. Prepared sections were mounted in Fluoromount (EMS) and examined using a Zeiss 102 microscope. To quantify the number of ChAT-positive axons, micrographs of the whole-mount retinas were taken and the total numbers of axon bundles were counted in an 800 × 1200 µm field adjacent to the optic disc.

Acknowledgments

We thank Q. Fu and M. Wu for technical assistance and Jose Luis de la Pompa for critical reading of the manuscript. This work was supported by the Silbermann Foundation and Retinal Research Foundation (L.G.) and the Margaret S. Pompa for critical reading of the manuscript. This work was supported by the Silbermann Foundation and Retinal Research Foundation (L.G.) and an NEI grant (RO1EY11930) and the Welch Foundation (W.H.K.).

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*Genes Dev.* 2001, 15:
Access the most recent version at doi:10.1101/gad.855301

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