Notch signaling regulates growth and differentiation in the mammalian lens

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ABSTRACT

The Notch signal transduction pathway regulates the decision to proliferate versus differentiate. Although there are a myriad of mouse models for the Notch pathway, surprisingly little is known about how these genes regulate early eye development, particularly in the anterior lens. We employed both gain-of-function and loss-of-function approaches to determine the role of Notch signaling in lens development. Here we analyzed mice containing conditional deletion of the Notch effector Rbpj or overexpression of the activated Notch1 intracellular domain during lens formation. We demonstrate distinct functions for Notch signaling in progenitor cell growth, fiber cell differentiation and maintenance of the transition zone. In particular, Notch signaling controls the timing of primary fiber cell differentiation and is essential for secondary fiber cell differentiation. Either gain or loss of Notch signaling leads to formation of a dysgenic lens, which in loss-of-function mice undergoes a profound postnatal degeneration. Our data suggest both Cyclin D1 and Cyclin D2, and the p27^{Kip1} cyclin-dependent kinase inhibitor act downstream of Notch signaling, and define multiple critical functions for this pathway during lens development.

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Introduction

Cellular organization into patterned structures is fundamental during animal development, with growth, patterning, morphogenesis and differentiation essential components of this process. Each event is spatiotemporally integrated, ensuring tissues and organs achieve proper size, shape and composition. Like other tissues and organs, vertebrate lens development requires careful coordination of these four components. Epithelial cells in the ocular lens undergo two temporally distinct modes of differentiation into fiber cells. First, primary fiber cells differentiate shortly after the lens invaginates from a placode to a lens pit and then into a lens vesicle. At this time, posterior lens progenitors closest to the central retina exit the cell cycle and initiate fiber cell differentiation, which is marked by the expression of alpha, beta, and gamma crystallin genes, and other fiber cell components. Secondary fiber cell differentiation directly follows, and is distinguished by a concerted migration of lens anterior epithelial layer (AEL) cells around the periphery to the equatorial region, wherein cells exit mitosis and migrate into the central lens. This equatorial region of the lens, where multiple signaling molecules converge on lens precursors, constitutes the transition zone, which remains the organizing center of lens fiber differentiation throughout the life of a vertebrate organism.

For both primary and secondary fiber cell differentiation, a highly conserved hierarchy of transcription factors orchestrates terminal differentiation into enucleated and organelle-deficient lens fiber cells. These final steps are critical for normal vision, as light must pass through an optically transparent lens to activate phototransduction within the retina. The transcription factors Pax6, Prox1, Maf, and Sox1 are essential regulators in the lens, since they directly regulate crystallin expression and fiber cell differentiation is blocked in their absence (Ashery-Padan et al., 2000; Cvekl et al., 1995; Glaser et al., 1994; Grindley et al., 1995; Kim et al., 1999; Nishiguchi et al., 1998; Ring et al., 2000; Wigle et al., 1999). These same factors, most notably Prox1, each promote the expression of cell cycle inhibitory molecules, including the Cyclin-dependent kinase inhibitors (CKIs) p27^{Kip1} (Cdkn1b) and p57^{Kip2} (Cdkn1c) (Wigle et al., 1999). CKIs have complex functions in the cell cycle, not only to inhibit Cyclin-CDK function, but also to promote S-phase in a context-dependent fashion (Besson et al., 2007). Lens cells lacking both p27^{Kip1} and p57^{Kip2} are unable to exit the cell cycle at the transition zone and fail to terminally differentiate and elongate, resulting in a propensity for apoptotic cell death via a p53-dependent pathway (Zhang et al., 1998). However, lens cell mitogens have remained elusive, either because they act redundantly or are broadly required throughout the body, thereby causing early embryonic lethality when mutated. Nonetheless, in vivo misexpression studies have pointed to Cyclin D1 (Ccnd1), Cyclin D2

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(*Ccnd2*), and *Cdk4* as likely targets of such a pro-mitogenic pathway (Gómez Lahoz et al., 1999).

FGF and BMP signaling at the transition zone are critical for lens fiber cell differentiation and survival (Beebe et al., 2004; Belecky-Adams et al., 2002; Faber et al., 2002; Robinson, 2006). Also, signaling through a Ras-MAPK pathway regulates some aspects of lens proliferation (Lovicu and McAvoy, 2001). Activated Ras signaling, by transgenic misexpression of dominant-active *H-Ras*, or the upstream ligand *Pdgfa*, causes over-proliferation of the lens epithelium (Reneker and Overbeek, 1996; Reneker et al., 2004). Conversely, transgenic expression of dominant-negative *H-Ras* impairs lens growth, thereby causing a small (microphthalmic) lens (Xie et al., 2006). Surprisingly, these perturbations in lens proliferation do not result in fiber cell defects, suggesting that other molecular pathway(s) coordinate the decision to proliferate versus differentiate.

The *Notch* signal transduction pathway is one of the major metazoan signaling networks. Canonical activation of this pathway occurs when a Notch receptor is engaged from a neighboring cell via the Delta-like (Dll) or Jagged (Jag) ligands. The Notch receptor undergoes proteolytic cleavage that liberates an intracellular domain (Notch[IC]), which translocates to the nucleus and acts in a transcriptional complex with Mastermind (Maml) and the Rbpj DNA-binding transcription factor (also known as RBP-Jκ1, CSL, or CBF-1) to activate Hairy-related transcriptional repressors (Fischer and Gessler, 2007; Ilagan and Kopan, 2007). Notch activation generally prevents differentiation and maintains progenitor or stem cell proliferation and is a classical mediator of lateral inhibition during cell fate determination (Bolós et al., 2007; Yoon and Gaiano, 2005). But *Notch* signaling has diverse, almost unlimited cellular outcomes, since it can regulate cell cycle progression, survival, fate determination, and morphogenesis in different organs and cellular contexts (Artavanis-Tsakonas et al., 1999; Thomas, 2005).

In the CNS and pancreas, disruption of *Notch* signaling causes premature progenitor cell differentiation, often leading to altered timing of differentiation of early-born cell types and a rapid depletion of the progenitor pool. Such phenotypes in the CNS, retina, and pancreas occur in mice lacking the *Notch* effector gene, *Hes1* (Hatakeyama et al., 2004; Ishibashi et al., 1995; Jensen et al., 2000; Kageyama et al., 2000; Lee et al., 2005; Tomita et al., 1996). In a recent study of frog lens induction, Ogino et al. demonstrated that a Delta1–Notch signal from the optic vesicle to the lens placode helps regulates the progression of lens induction via Otx2 and Rbpj-mediated activation of Foxe3 transcription (Ogino et al., 2008). Intriguingly, *Hes1* mutant mice also display defects in early lens development that range from complete loss to a microphthalmic lens, with reduced proliferation as early as the lens pit stage (Lee et al., 2005; Tomita et al., 1996). Recently, a conditional deletion of *Rbpj* in the developing lens was reported, resulting in a smaller lens and possible upregulation of p57[Kip2] (Jia et al., 2007). The minor alterations in fiber cell differentiation reported in this study are inconsistent with the *Hes1* mutant phenotype, and thus do not fully resolve the question of what processes *Notch* signaling regulates in the lens.

Here, we evaluate the consequences of both loss and gain of *Notch* signaling during mammalian lens development. Mice lacking *Notch* signaling, through tissue-specific removal of *Rbpj*, exhibit accelerated primary fiber cell differentiation and hypoproliferation accompanied by reduced levels of Pax6, Cyclin D1, and Cyclin D2. These defects result in the essentially complete loss of the lens (aphakia) in postnatal *Rbpj* conditionally mutant mice. Moreover, mice with constitutive *Notch* signaling through tissue-specific expression of the Notch1[IC], show abnormal lens morphogenesis, hyperproliferation of the AEL, and inappropriate maintenance of Pax6 and other AEL-expressed genes. This causes severely delayed primary fiber cell differentiation. In both genetic manipulations of *Notch* signaling, the transition zone is malformed and secondary fiber cell differentiation is lost. Together, our data demonstrate that *Notch* signaling is essential for lens growth and differentiation.

Materials and methods

Animals

Rosa26[Notch1IC] mice were described previously (Murtaugh et al., 2003) and maintained as homozygotes. The P0-3.9-GFPCre construct was generated by replacing the NotI fragment containing the lacZ reporter from P0-3.9-lacZ (Zhang et al., 2002) with an XhoI–XbaI fragment, containing GFPCre from pBS-592 (Le et al., 1999). These regulatory elements are largely overlapping those of *Le-Cre* (including the *EE*). The linearized insert was injected into the male pronuclei of fertilized FVB eggs using standard techniques. The *P0-3.9-GFPCre* line is maintained on an FVB background and genotyped using a standard PCR protocol. *Rbpj*[tm1Hon] mice (termed *Rbpj*[CKO]), were generated by Han et al., and maintained on a 129/SvJ background and genotyped as described (Han et al., 2002). *Le-Cre* mice, generated by Ashery-Padan et al., were maintained on a CD-1 background and PCR genotyped as described (Ashery-Padan et al., 2000). Images of adult heads or eyeballs were captured with a Leica dissecting microscope and Optronics digital camera.

Tissue analyses

Embryonic and postnatal tissue was fixed in 4% paraformaldehyde/PBS for 15 min–1 h at 4 °C and processed by stepwise sucrose/PBS incubation for 10 μm frozen sections in OCT by standard techniques. Primary antibodies used include anti-BrdU (BD Laboratories clone B44 1:100 or Serotec clone BU1/75 1:500), anti-cleaved PARP (Cell Signaling 1:500), anti-Cre (Novagen 1:5000), anti-Cyclin D1 (Neomarkers SP4 1:100; Sigma DCS-6 1:100 or Santa Cruz 72-13G 1:500), anti-Cyclin D2 (Santa Cruz 34B1-3 1:200), anti-E cadherin (Zymed ECCD-2 1:500), anti-Foxe3 (a gift from Peter Carlsson 1:1000), anti-beta crystallin (a gift from Richard Lang 1:8000), anti-gamma crystallin (Santa Cruz 1:1000), anti-GFP (Molecular Probes 1:1000 or Abcam 1:1000), anti-Hes1 (1:1000), anti-Jagged1 (Santa Cruz 1:1000), anti-p27[Kip1] (BD Laboratories Clone 57 1:100 or Assay Designs 1:500), anti-p57[Kip2] (Abcam 1:500 or Santa Cruz 1:50), anti-Pax6 (Covance 1:1000 or DSHB 1:20), anti-Prox1 (Covance 1:1000 or Chemicon 1:2000), anti-Pitx3 (a gift from Marten Smidt 1:1000), anti-Six3 (a gift from Guillermo Oliver 1:1000), anti-Sox1 (Affinity BioReagents 1:500), and anti-Sox2 (Chemicon 1:500). Detailed staining protocols are available upon request and generally followed those in Lee et al. (2005) and Zhang et al. (2003). Secondary antibodies were generated in donkey or goat versus the appropriate species and directly conjugated with Cy3 (Jackson Immunologicals), Alexa Fluor 488, Alexa Fluor 594 (Molecular Probes) or biotinylated (Jackson Immunologicals) and sequentially labeled with streptavidin Alexa 488 or 594 (Molecular Probes). Labeled sections were visualized with a Zeiss fluorescent microscope equipped with either a Leica or Zeiss camera and Apotome deconvolution device. Whole-mount or cryosection in situ hybridization was performed as described (Brown et al., 1998) using an *Rbpj* digoxygenin-labeled antisense riboprobe. For S-phase analyses, BrdU (Sigma) was injected intraperitoneally as described (Mastick and Andrews, 2001) and animals were sacrificed 1.5–4 h later for tissue processing. Tissue sections were treated with 2N hydrochloric acid prior to standard antibody staining. TUNEL staining was performed using the in situ cell death detection kit, according to the manufacturer's instructions (Roche). Standard histologic staining of frozen or paraffin embedded sections was also performed. All images were processed using Axiovision (v5.0) and/or Adobe Photoshop software (v7.0) and manipulated electronically to adjust brightness and contrast as well as pseudocoloring.

Cell counting

Tissue sections, separated by at least 60 μm, were antibody-stained and counted using NIH ImageJ or Axiovision software. Between 3–5 animals were analyzed per genotype and age and at least two