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Presenilin gene function and Notch signaling feedback regulation in the developing mouse lens

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ABSTRACT

Presenilins (Psen1 and Psen2 in mice) are polytopic transmembrane proteins that act in the γ-secretase complex to make intra-membrane cleavages of their substrates, including the well-studied Notch receptors. Such processing releases the Notch intracellular domain, allowing it to physically relocate from the cell membrane to the nucleus where it acts in a transcriptional activating complex to regulate downstream genes in the signal-receiving cell. Previous studies of Notch pathway mutants for *Jagged1*, *Notch2*, and *Rbpj* demonstrated that canonical signaling is a necessary component of normal mouse lens development. However, the central role of *Psens* within the γ-secretase complex has never been explored in any developing eye tissue or cell type. By directly comparing *Psen* single and double mutant phenotypes during mouse lens development, we found a stronger requirement for *Psen1*, although both genes are needed for progenitor cell growth and to prevent apoptosis. We also uncovered a novel genetic interaction between *Psen1* and *Jagged1*. By quantifying protein and mRNA levels of key Notch pathway genes in *Psen1/2* or *Jagged1* mutant lenses, we identified multiple points in the overall signaling cascade where feedback regulation can occur. Our data are consistent with the loss of particular genes indirectly influencing the transcription level of another. However, we conclude that regulating Notch2 protein levels is particularly important during normal signaling, supporting the importance of post-translational regulatory mechanisms in this tissue.

1. Introduction

Cellular changes in growth, morphogenesis, and differentiation all contribute to tissue composition and shape, but ultimately, influence functionality. The vertebrate ocular lens is a prime example of this complex process. This spheroidal, transparent tissue must achieve precise size and curvature during development to focus images onto the retina for proper vision. Lens development initiates at the surface ectoderm in a region overlaying the optic vesicle, known as the presumptive lens ectoderm (PLE) (McAvoy et al., 1999). Fundamental experiments in frog embryos demonstrated that lens induction requires signals from the developing optic vesicle (Spemann, 1938). PLE cells respond by thickening into the lens placode, which folds into a pit shape that eventually pinches off from the surface ectoderm to give rise to the lens vesicle, a uniform, hollow structure comprised of proliferating progenitor cells. This is followed by two temporal waves of fibergenesis that both begin prenatally. Primary fibergenesis initiates when posterior lens vesicle cells elongate across the lumen and differentiate. At this time, anterior vesicle cells remain proliferative and coalesce to create the anterior epithelial layer (AEL). During secondary fibergenesis, AEL cells move peripherally through the germinative zone where they become postmitotic and progress into the transition zone for terminal differentiation (Lovicu and Robinson, 2004).

A variety of intrinsic and extrinsic factors are essential for proper lens growth and fiber cell differentiation. Some key transcription factors include: *Pax6*, *Sox2*, *FoxE3*, *Prox1*, and *c-Maf* (Blixt et al., 2000; Chow et al., 1999; Hill et al., 1991; Kamachi et al., 1995; Kim et al., 1999; Wigle et al., 1999). These factors regulate lens growth, fiber cell differentiation, elongation, cell-cycle exit and organelle-clearing. Moreover, lens formation also relies on many signaling factors. Classic lens inversion experiments performed in chicks embryos offered the first clue that signals in the aqueous humor or vitreous regulate lens fiber elongation and polarity (Coulombre and Coulombre, 1963). Much later some of the signals were identified, including *Fgfs*, *Wnts*, *Bmp*, and

Abbreviations: CKO, "conditional knock-out" allele; ICD, intracellular domain; NEXT, Notch extracellular truncation; PLE, presumptive lens ectoderm; AEL, anterior epithelial layer; E0.5, embryonic day 0.5; H&E, hematoxylin and eosin; OCT, optimal cutting temperature; RQ, relative quantification; IHC, Immunohistochemistry

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Notch (Cain et al., 2008; Chamberlain and McAvoy, 1987; Faber et al., 2002; Garcia et al., 2011; Jia et al., 2007; Le et al., 2009; Rowan et al., 2008; Stump et al., 2003). One pathway with an essential role in secondary fibergenesis is Notch signaling (Jia et al., 2007; Rowan et al., 2008). Canonical cell-cell signaling enables ligands on the surface of one cell to engage with a Notch receptor expressed on the neighboring cell (Kopan and Ilagan, 2009; Kovall et al., 2017). There are two families of Notch ligands: *Deltalike (Dll)* and *Jagged (Jag)*. Upon ligand binding, the Notch receptor protein undergoes a conformational change which exposes its negative regulatory region for cleavage by proteases. This fundamental activation step ultimately releases the Notch intracellular domain (NICD) (Schroeter et al., 1998). Receptor cleavage is sequentially mediated by two different protein complexes. First, ADAM secretase removes the large extracellular domain (Brou et al., 2000; Mumm et al., 2000) and then γ-secretase cuts within the transmembrane region to release NICD from the plasma membrane (De Strooper et al., 1999). The γ-secretase complex is comprised of four protein subunits: Nicastrin, PEN2, APH1, and the catalytic subunit Presenilin (Psen). The two mouse *Psen* genes, *Psen1* and *Psen2*, encode proteins that catalyze substrate proteolysis, however each γ-secretase complex contains only one Psen paralog (Donoviel et al., 1999; Struhl and Greenwald, 1999). After cleavage and release, the NICD first binds to the DNA-binding protein Rbpj and subsequently a cofactor, Mastermind. This complex transcriptionally activates downstream genes, such as the *Hes* and *Hey* genes (Iso et al., 2003; Kovall, 2007; Kuroda et al., 1999; Tamura et al., 1995). Previously, we demonstrated that the activities of Notch pathway genes *Jag1*, *Notch2*, and *Rbpj* are required for lens development (Le et al., 2009; Rowan et al., 2008; Saravanamuthu et al., 2012). Given the pivotal role of the γ-secretase complex in canonical Notch signaling, we wished to explore the role(s) of *Psen* genes within the context of mouse lens development.

Psen1 mutations cause embryonic lethality, whereas *Psen2* mutants are adult viable, illustrating the vastly different requirements for γ-secretase complexes containing either Psen protein (Herreman et al., 1999; Shen et al., 1997). There is also evidence of distinct subcellular distribution of Psen1- versus Psen2-containing γ-secretase complexes, which correlates with differential substrate specificity (Sannerud et al., 2016). For the Notch receptors, NICD generation is more heavily dependent on *Psen1* than *Psen2* (Zhang et al., 2000). However, for the lens, the extent to which Psen1- versus Psen2-containing γ-secretase complexes regulate Notch receptor activation is unknown. Moreover, given that γ-secretase can cleave up to 90 different proteins (Haapasalo and Kovacs, 2011), it is possible that *Psen* loss may have greater consequences during lens development, than do other Notch pathway mutants. To address these questions, we utilized the Le-Cre driver and $Psen1^{CKO/CKO}$;$Psen2^{-/-}$ mouse stocks to generate an allelic series during lens formation that includes *Psen1/2* double mutants, which block all Notch receptor activation. Here we report the lens phenotypes of this *Psen1/2* allelic series. We found that although the *Psen1/2* double mutant lenses complete primary fibergenesis, and initiate secondary fibergenesis, starting at E14.5 proliferation becomes dramatically reduced, *FoxE3* is downregulated and there is a significant increase in apoptosis. Postnatally, *Psen1/2* double mutants progressively lose lens tissue, exhibiting aphakia by P21. We also uncovered a genetic interaction between *Psen1* and *Jag1*, wherein Le-Cre;$Psen1^{CKO/+}$;$Jag1^{CKO/+}$ adult mice display synergistic lens phenotypes. To understand the basis for this interaction, we measured the mRNA and protein levels of relevant Notch pathway genes in each other's mutant lens tissue. We found that at the onset of secondary fibergenesis, Notch2 protein levels are particularly sensitive to both *Jag1* and *Psen1/2* activity. Together, these data demonstrate Presenilins play a critical role in lens growth and homeostasis. We conclude that while Notch signaling is controlled at both the level of transcription and post-translation, in the lens it is the latter mechanism that is utilized for feedback regulation.

2. Materials and methods

2.1. Animals

$Psen1^{tm1Shn/tm1Shn}$;$Psen2^{tm1Bdes/tm1Bdes}$ ($Psen1^{CKO/CKO}$;$Psen2^{-/-}$) mice, $Jagged1^{tm1JLew/tm1Lew}$ mice ($Jag1^{CKO/CKO}$), $Notch1^{tm2Rko}/^{tm2Rko}$ ($Notch1^{CKO/CKO}$) mice, $Notch2^{tm3Grid/tm3Grid}$ ($Notch2^{CKO/CKO}$) mice and $Notch1^{CKO/CKO}$; $Notch2^{CKO/CKO}$ mice were each maintained on a mixed 129-C57BL/6 background and genotyped as described (Beglopoulos et al., 2004; Brooker et al., 2006; McCright et al., 2006; Yang et al., 2004). $Hes1^{tm1Hojo}$ mice ($Hes1^{CKO/CKO}$) were maintained on a CD-1 background and genotyped as described (Kita et al., 2007). Le-Cre Tg/+ mice were maintained on a FVB/N background and PCR genotyped as described (Ashery-Padan et al., 2000). MLR10-Cre Tg/+ mice were maintained on a FVB/N background and PCR genotyped as described (Zhao et al., 2004). Our Le-Cre Tg/+ line does not exhibit DNA damage pathway activation (Loonstra et al., 2001), shown by anti-H2A.X western blotting (Supplemental Fig. 2F). We also monitored Pax6 mRNA levels among all genotypes produced in different litters, and found no changes correlating with inheritance of the Le-Cre transgene alone.

Breeding schemes for all analyses mated one Cre Tg/+ mouse (Le- or MLR10-Cre) to one homozygous for a conditional allele (e.g. $Psen1^{CKO/CKO}$ or $Psen1^{CKO/CKO}$;$Psen2^{-/-}$) to create F_1 Cre;gene of interest$^{CKO/+}$ heterozygous mice. All embryonic or postnatal analyses used F_2 generation animals generated by timed matings between Cre;gene of interest$^{CKO/+}$ X gene of interest$^{CKO/CKO}$ mice. The resulting lens phenotypes occurred at expected Mendelian recessive ratios, completely correlating with the loss of gene of interest. Le-Cre;$Jag1^{CKO/+}$;$Psen1^{CKO/+}$ double heterozygote phenotypic analyses also used F_2 mice, generated in timed matings between Le-Cre;$Psen1^{CKO/+}$ X $Jag1^{CKO/CKO}$ mice, or Le-Cre;$Jag1^{CKO/+}$ X $Psen1^{CKO/CKO}$ mice. Finally, we also analyzed F_2 litters containing Le-Cre;$Jag1^{CKO/+}$;$Psen1^{CKO/+}$;$Psen2^{+/-}$ triple heterozygotes by timed matings of either Le-Cre;$Psen1^{CKO/+}$;$Psen2^{+/-}$ F_1 males and $Jag1^{CKO/CKO}$ females, or Le-Cre;$Jag1^{CKO/+}$ F_1 males to $Psen1^{CKO/CKO}$;$Psen2^{-/-}$ females.

The embryonic age was based on vaginal plug detection at day E0.5. The heads of anesthetized adult mice were imaged with a Leica MZ8 dissecting microscope, DFC290 HD camera and Leica LAS V4.2 software. Standard H&E paraffin histology of P21 eyes was also performed and those data gathered using a Nikon eclipse E800 scope, Olympus DP74-CU camera and cellSens Dimension software (v1.17). All mice were housed and cared for in accordance with the guidelines provided by the National Institutes of Health, Bethesda, Maryland, and the Association for Research in Vision and Ophthalmology, and conducted with approval and oversight from the Cincinnati Childrens and UC Davis Institutional Animal Care and Use Committees.

2.2. Immunohistochemistry and cell counting

Embryonic tissue was fixed in 4% paraformaldehyde/PBS for 1hr on ice, processed by stepwise sucrose/PBS incubation and embedded in OCT, then 10 μm frozen sections were generated for marker analyses as described in (Brown et al., 1998). Anti-BrdU labeling was performed as in Le et al. (2006). The primary antibodies used were mouse anti-BrdU (1:100, Becton Dickinson Cat#:347580), rat anti-Ccnd2 (1:200, Santa Cruz Cat#: sc-452), rat anti-Cdh1 (1:500, Invitrogen Cat#:13–1900), rabbit anti-cleaved PARP (1:500, Cell Signaling Cat#:9544), rabbit anti-Cryba1 (1:5000, gift from Richard Lang), goat anti-FoxE3 (1:200, Santa Cruz Cat#: sc-48162-discontined), goat anti-Jag1 (1:200, Santa Cruz Cat#: sc-6011-discontinued), rabbit anti-Prox1 (1:5000, Millipore Cat#: AB5475), rabbit anti-Psen1 (1:100, Santa Cruz Cat#: sc-7860-discontinued). Sections were subsequently incubated with directly conjugated Alexafluor secondary antibodies (1:400, Jackson ImmunoResearch or Molecular Probes) or biotinylated secondary antibodies (1:500, Jackson ImmunoResearch or ThermoScientific) followed by Alexafluor conjugated streptavidin (1:500, Jackson