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Notch signaling regulates endocrine cell specification in the zebrafish anterior pituitary

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ABSTRACT

The vertebrate pituitary gland is a key endocrine control organ that contains six distinct hormone secreting cell types. In this study, we analyzed the role of direct cell-to-cell Delta–Notch signaling in zebrafish anterior pituitary cell type specification. We demonstrate that initial formation of the anterior pituitary placode is independent of Notch signaling. Later however, loss of Notch signaling in *mind bomb* (*mib*) mutant embryos or by DAPT treatment leads to increased numbers of lactotropes and loss of corticotropes in the anterior pars distalis (APD), increased number of thyrotropes and loss of somatotrope cell types in the posterior pars distalis (PPD), and fewer melanotropes in the posterior region of the adenohypophysis, the pars intermedia (PI). Conversely, Notch gain of function leads to the opposite result, loss of lactotrope and thyrotrope cell specification, and an increased number of corticotropes, melanotropes, and gonadotropes in the pituitary. Our results suggest that Notch acts on placodal cells, presumably as a permissive signal, to regulate progenitor cell specification to hormone secreting cell types. We propose that Notch mediated lateral inhibition regulates the relative numbers of specified hormone cell types in the three pituitary subdomains.

Introduction

Several long-range signals, including Nodals, Hedgehogs (Hh), Bone Morphogenetic Proteins (BMP), Fibroblast Growth Factors (FGF), and Wnts, regionalize and pattern the anterior pituitary (also called adenohypophysis) and instruct pituitary cells about their positions and later cell fates (Dasen and Rosenfeld, 2001; Dutta et al., 2005; Ericson et al., 1998; Herzog et al., 2003; Sbrogna et al., 2003; Treier et al., 1998). However, it remains unclear how pituitary cells acquire competence to respond to these signals or how they adopt different cell fates in the three pituitary subdomains, even though they are exposed to similar concentrations of particular long-range signals. The potential functions of short-range, cell-to-cell signals, for example by Delta ligands and Notch receptors that mediate lateral inhibition, for pituitary cell specification have not been tested.

The vertebrate anterior pituitary develops from unspecified ventral ectoderm cells at the anterior midline of the neural plate border (Couly and Le Douarin, 1988; Eagleson and Harris, 1990). Mouse pituitary expresses genes encoding homeodomain transcription factors such as Pitx1 and Pitx2, and in zebrafish, pituitary precursor cells and pituitary placode are demarcated by *pitx3* and *dlx3b* (Dutta et al., 2005). The visible placode forms by mid-somitogenesis stages (Glasgow et al., 1997). The anterior pituitary placode is later regionalized into three distinct subdomains: the anterior pars distalis (APD), the adjacent posterior pars distalis (PPD), and the posterior pars intermedia (PI) (Herzog et al., 2003). The 6 hormone secreting cell types of the pituitary are distinguished by their morphologies, by the hormones they secrete (Bentley, 1998), and by their locations in subdomains along the anteroposterior axis (Dasen and Rosenfeld, 2001; Herzog et al., 2003). Two cell types are located in the APD. One cell type expresses *pomc* that encodes Proopiomelanocortin (POMC). This pre-proprotein is differentially processed to form several functional peptides, such as Adrenocorticotropin (ACTH) that is secreted by corticotrope cells in the APD (Dasen and Rosenfeld, 2001). The second cell type in the APD are lactotropes that secrete Prolactin (Prl; Herzog et al., 2003). Three cell types are located in the PPD, thyrotropes that express *thyroid-stimulating hormone β subunit* (*tsh-β*), somatotropes that express *growth hormone* (*gh*; Herzog et al., 2003), and gonadotropes that secrete Follicle-Stimulating Hormone (FSH) or Luteinizing Hormone (LH). Like TSH, these hormones are glycoproteins composed of a common α-glycosylated subunit (α-GSU; encoded by *cga*) and a hormone specific β-subunit (Dasen and

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Rosenfeld, 2001). In the posterior most region of the anterior pituitary placode (PI), melanotropes express *pomc* and secrete the posttranslational POMC product, α-Melanocyte-Stimulating Hormone (α-MSH).

Recently, we have shown that long-range Hh signal specifies pituitary characteristics in a field of undifferentiated placodal precursor cells (Dutta et al., 2005). In addition, varying concentrations of Hh signal contribute to the regionalization of the pituitary placode into three subdomains (Herzog et al., 2003; Sbrogna et al., 2003). Presently, it is unclear how different cell fates are specified within the subdomains of the pituitary placode. During development of many organs, differentiated cell types emerge from a common field of progenitor cells by a mechanism termed "lateral inhibition". During this process cells initially co-express Notch receptors and Delta ligands. The extracellular domain of the Delta ligand binds to the Notch receptor on adjacent cell surfaces. This interaction activates bidirectional signaling that ultimately results in unidirectional signaling between adjacent cells and adoption of distinct cell fates (Schweisguth, 2004). This direct cell–cell interaction through Delta–Notch signaling contributes to cell differentiation in a wide variety of organisms (Artavanis-Tsakonas et al., 1999).

Delta ligands are single pass transmembrane proteins present on the cell surface. Notch receptors are also transmembrane proteins with extracellular and intracellular domains. Furin cleaves the Notch extracellular domain and the two resulting peptides form a Notch heterodimer on the cell surface. Binding of Delta to Notch extracellular domain on neighboring cell surfaces triggers TACE metalloprotease mediated cleavage at an extracellular site of the Notch transmembrane domain (Brou et al., 2000). This cleavage facilitates removal of the Notch extracellular domain from the neighboring cell. Experiments in *Drosophila* and *Xenopus* indicate that ubiquitinylation of Delta and subsequent endocytosis of the Delta–Notch heterodimers play a key role in the activation of the Notch signaling cascade (Lai, 2002; Parks et al., 2000). Similarly, *mind bomb* (*mib*) mutant zebrafish embryos lack functional ubiquitin ligase, are unable to activate Notch signaling, and display a neurogenic phenotype (Itoh et al., 2003). Ubiquitinylation and sequestration of the Delta–Notch heterodimers activate cleavage of membrane bound Notch by the γ-secretase complex that releases the Notch intracellular domain (NICD). In the nucleus, NICD forms a functional transcription complex in association with Su(H), CBF1, and lag-1 proteins that subsequently regulates Notch target gene expression (De Strooper et al., 1999). Pharmacological treatment with *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-(*S*)-phenylglycine *t*-butyl ester (DAPT), a γ-secretase inhibitor, specifically prevents NICD formation (Dovey et al., 2001) and blocks Notch signal transduction thus leading to typical phenotypes consistent with loss of Notch signaling in zebrafish and *Drosophila* (Geling et al., 2002).

Prop1 controls pituitary progenitor cell differentiation and a hypomorphic mutation of *Prop1* leads to pituitary hypoplasia by an unknown mechanism. A recent study in *Prop1* mutant mice showed that *Notch2* expression is almost entirely absent in the pituitary, suggesting an indirect role of Notch in pituitary development (Raetzman et al., 2004). Overexpression of Notch2 delays gonadotrope development in the anterior lobe (Raetzman et al., 2006). Moreover, the transcriptional repressor *Hes1* regulates survival and proliferation of Rathke's Pouch precursor cells and is necessary for anterior lobe size. *Hes1* is also necessary for melanotrope cell differentiation in the intermediate lobe (Raetzman et al., 2007). These studies suggest that downstream targets of Notch signaling regulate pituitary cell survival, proliferation, and differentiation in the anterior pituitary. However, direct functional studies of Notch signaling in pituitary cell type differentiation have not been carried out in all of the pituitary subdomains and it remains unclear which other cell types differentiate from *delta* or *notch* expressing progenitor cells. In the present study, we analyzed the role of Notch signaling in specification of zebrafish adenohypophysis cells because of their limited number, defined locations within pituitary placode subdomains, and experimental and genetic accessibility.

We show a direct role of the Notch signaling pathway in pituitary cell numbers and specification. We found that pituitary placode cells express *delta* and *notch* during mid-somitogenesis stages, consistent with their hypothesized role in cell differentiation or specification. To analyze Notch function, we characterized pituitary development in Notch gain and loss of function experiments. Altered Notch signaling does not affect pan-pituitary *lim3* and *pitx3* expression, suggesting that this short-range signal is not involved in the initial formation of the pituitary preplacode or placode. However, once the placode has formed, Notch restricts specification of lactotropes and thyrotropes. Notch is also necessary and sufficient for specification of corticotropes and melanotropes, and it acts as a permissive factor for the differentiation of somatotropes. Block of Notch signaling at various developmental stages by DAPT suggests that Notch is necessary for cell type specification only during early somitogenesis stages, even though it is expressed in the pituitary until later stages.

Materials and methods

Husbandry

Zebrafish (*Danio rerio*) embryos were obtained and maintained by standard procedures (Westerfield, 2007). Except for heat-shock experiments, embryos were maintained at 28.5 °C. Embryos were staged by hours postfertilization (h) based on morphological staging criteria (Kimmel et al., 1995). Mutant strains used in this study were *mind bomb* (*mib*^{ta52b}; Itoh et al., 2003 and *after-eight aei*^{tr233}; Holley et al., 2000). We used two transgenic lines *Tg*(*UAS:myc-Notch1a-intra*)^{kca3} and *Tg*(*hsp70l:Gal4*)*1.5*^{kca4} (Scheer and Campos-Ortega, 1999). Embryos carrying either one or both of the transgenes were obtained either by incrossing *Tg*(*hsp70l:Gal4*)*1.5*^{kca4} carriers or by crossing *Tg*(*UAS:myc-Notch1a-intra*)^{kca3} with *Tg*(*hsp70l:Gal4*)*1.5*^{kca4} carriers (we abbreviate the transgenic lines as *Tg*(*UAS:Notch1a*) and *Tg*(*hsp70:Gal4*)).

Whole-mount mRNA in situ hybridization and immunohistochemistry

We analyzed gene expression by mRNA in situ hybridization with one or two mRNA probes (Hauptmann and Gerster, 2000). Antisense mRNA was *in vitro* synthesized using digoxygenin or fluorescein RNA labeling kits (Roche). For double in situ hybridization, the digoxygenin probe was detected using anti-digoxygenin-AP and NBT/BCIP as described earlier (Dutta et al., 2005). Fluorescein labeled probes were detected using anti-fluorescein-AP and INT/BCIP. We used probes for *pitx3* and *lim3* to label pituitary precursors and placode; and we used antisense mRNA probes for *deltaA*(*dlA*), *deltaB* (*dlB*), *deltaC*(*dlC*), *deltaD*(*dlD*), *notch1a*, *notch1b*, *notch5*, and *notch6* expression (Bierkamp and Campos-Ortega, 1993; Haddon et al., 1998; Westin and Lardelli, 1997). To analyze cell specification in the APD (lactotropes, corticotropes), PPD (somatotropes, thyrotropes, gonadotropes), and PI (melanotropes), we labeled pituitary hormone cell types at Prim-5 (24 h) and Protruding mouth stage (72 h) and used *prl*, *gh*, *pomc*, *tsh-β* and *cga* (previously *α-gsu*) mRNA probes (Herzog et al., 2003; Nica et al., 2004).

DAPT block of Notch function

We dechorionated wild-type embryos using 0.1 mg/ml pronase in embryo medium at room temperature. 100 mM stock solution of the γ-secretase inhibitor (DAPT) was prepared in DMSO, stored at −20 °C, and diluted to 100 μM in embryo medium. Dechorionated embryos were DAPT incubated in small Petri-dishes (3.5 cm diameter) at 28.5 °C in the dark. Embryos (n=30) were incubated in 3 ml of 100 μM DAPT (Geling et al., 2002) at different developmental stages (shield, 6 h; Bud, 10 h; 6-somite, 12 h; 14-somoite, 16 h; 18-somite, 18 h; Prim-22, 36 h; Long pec, 48 h; Pec fin, 60 h). Control embryos were incubated in embryo medium with DMSO (3 μL DMSO in 2997 ml of embryo medium).

DAPT treatment was stopped at different stages of development (Prim-5 (24 h) stage; Protruding mouth, (72 h) stage) and embryos were rinsed several times in DMSO/embryo medium to remove DAPT completely. After rinsing, embryos were raised in embryo medium at 28.5 °C until 72 h. Embryos were fixed at Prim-5 (24 h) and Protruding mouth (72 h) stages in 4% paraformaldehyde and analyzed by in situ hybridization and antibody labeling.

Heat-shock transactivation of Tg(UAS:myc-Notch1a-intra)

Groups of 5 to 10 transgenic embryos were heat shocked for 10 min at 40 °C in 0.25 ml PCR tubes using a thermocycler (PTC 200, MJ Research) heat block, which was