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Notch signaling regulates midline cell specification and proliferation in zebrafish

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Abstract

Notochord and floor plate cells are sources of molecules that pattern tissues near the midline, including the spinal cord. Hypochord cells are also found at the midline of anamniote embryos and are important for aorta development. Delta–Notch signaling regulates midline patterning in the dorsal organizer by inhibiting notochord formation and promoting hypochord and possibly floor plate development, but the precise mechanisms by which this regulation occurs are unknown. We demonstrate here that floor plate and hypochord cells arise from distinct regions of the zebrafish shield. Blocking Notch signaling during gastrulation entirely prevented hypochord specification but only reduced the number of floor plate cells that developed compared to control embryos. In contrast, elevation of Notch signaling at the beginning of gastrulation caused expansion of hypochord at the expense of notochord, but floor plate was not affected. A cell proliferation assay revealed that Notch signaling maintains dividing floor plate progenitors. Together, our results indicate that Notch signaling regulates allocation of appropriate numbers of different midline cells by different mechanisms.

Keywords: Delta; Notch; Notochord; Hypochord; Floor plate; Midline; Zebrafish

Introduction

The vertebrate embryonic midline is an important source of signaling molecules that pattern surrounding tissues during development. Midline structures include the floor plate of the ventral spinal cord, mesodermal notochord and, in anamniotes, the hypochord, a row of cells between the notochord and dorsal aorta. Secretion of Sonic hedgehog (Shh) by notochord and floor plate patterns the ventral spinal cord and somites (Echelard et al., 1993; Krauss et al., 1993; Marti et al., 1995; Roelink et al., 1994; Stickney et al., 2000; Tanabe and Jessell, 1996), and hypochord cells produce molecules such as VEGF that are important for dorsal aorta formation (Cleaver and Krieg, 1998). Fate mapping experiments in amphibian, chick and mouse embryos showed that midline precursor cells occupy overlapping territories within the dorsal organizer and gene expression patterns are consistent with the idea that floor plate and notochord precursors arise from a common region and subsequently separate to become the notochord and floor plate (Catala et al., 1996; Charrier et al., 1999; Gont et al., 1993; Le Douarin and Halpern, 2000; Ruiz i Altaba and Jessell, 1992; Ruiz i Altaba et al., 1995; Schoenwolf and Sheard, 1990; Selleck and Stern, 1991; Spemann, 1938; Teillet et al., 1998; Wilson and Beddington, 1996). Similar studies in zebrafish embryos also showed that notochord and floor plate precursors are close to one another in the dorsal organizer, or shield, although different fate maps showed slight differences in their arrangement (Ekker et al., 1995; Etheridge et al., 2001; Latimer et al., 2005; Melby et al., 1996; Shih and Fraser, 1995). The organizer region of anamniotes also gives rise to hypochord (Keller, 1975; Latimer et al., 2002), and our previous fate mapping placed their origin at the lateral edges of the shield, close to some notochord precursors (Latimer et al., 2002). These observations raised the possibility that cell–cell signaling among midline precursors mediates fate decisions that ultimately result in an appropriate number of notochord, floor plate and hypochord cells.

Cell–cell signaling mediated by the Delta ligand–Notch receptor signaling pathway often regulates fate decisions among neighboring cells in invertebrates and vertebrates (Artavanis-

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Tsakonas et al., 1999; Greenwald, 1998). In zebrafish, Notch pathway genes are expressed in or near the shield region during gastrulation and are later in the notochord, floor plate and hypochord (Appel et al., 1999, 2003; Bierkamp and Campos-Ortega, 1993; Dornseifer et al., 1997; Haddon et al., 1998; Latimer et al., 2002; Westin and Lardelli, 1997). Many of these genes are important for midline development (Appel et al., 1999, 2003; Julich et al., 2005; Latimer et al., 2002). We previously found that Delta–Notch signaling is required for an appropriate number of floor plate and hypochord cells and that Notch activity inhibits notochord marker expression (Appel et al., 1999, 2003; Latimer et al., 2002). These results indicated that midline precursors may undergo cell fate decisions mediated by the Delta–Notch pathway, with Notch activity promoting floor plate or hypochord, or both, at the expense of notochord. To clarify the role of Notch signaling in midline development, we extended our fate map analysis of the zebrafish shield region and performed a series of experiments to inhibit or potentiate Notch signaling at different times. Our results lead us to propose that Notch signaling has at least two distinct functions in the formation of midline cells in zebrafish. First, during gastrulation, it induces a subset of midline precursors at the edges of the shield to develop as trunk hypochord. Second, it promotes proliferation but not specification of floor plate precursors.

Materials and methods

Embryos

Embryos were collected from single pair matings of fish raised in the Vanderbilt University Zebrafish Facility, raised at 28.5°C in embryo medium (15 mM NaCl, 0.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.15 mM KH_2PO_4, 0.05 mM NH_2PO_4, 0.7 mM $NaHCO_3$) and staged according to hours post-fertilization (hpf) and morphological criteria (Kimmel et al., 1995). *Tg(hsp70l:XdnSu(H)myc)^{vu21}* lines were created in our laboratory (Latimer et al., 2005). *Tg(hsp70l:GAL4)1.5^{kca4}* and *Tg(UAS:myc-notch1a-intra)* lines have been described previously (Scheer and Campos-Ortega, 1999; Scheer et al., 2002). Transgenic embryos were raised at 28.5°C until specified times when they were heat-shocked. *Tg(hsp70l:XdnSu(H)myc)^{vu21}* transgenic embryos were obtained from crosses between hemizygous *Tg(hsp70l:XdnSu(H)myc)^{vu21}* and wild-type adults, and, after heat shock, approximately one-half of the embryos expressed XdnSu(H)myc as determined by anti-myc immunohistochemistry and were identifiable by their morphological phenotype, which is similar to *mind bomb* (*mib*) mutant embryos (Itoh et al., 2003). *Tg(hsp70l:GAL4);Tg(UAS:myc-notch1a-intra)* transgenic embryos were obtained from crosses between homozygous *Tg(hsp70l:GAL4)1.5^{kca4}* and hemizygous *Tg(UAS:myc-notch1a-intra)^{kca3}* adults. Approximately one-half of the embryos from a clutch of heat-shocked embryos failed to develop eyes, which we associated with expression of the *notch1a* intracellular domain (NICD) by determining that only those embryos with the eyeless phenotype expressed the myc epitope, as determined by immunohistochemistry (data not shown). The remaining embryos had no obvious morphological defects and were used as control embryos. *Tg(β-actin:mgfp)^{vu119}* embryos expressing membrane localized GFP uniformly were described previously (Cooper et al., 2005).

Heat shock experiments

All heat shocks were performed by placing embryos into a pre-warmed beaker of 40 ml embryo medium in a 39°C water bath. Embryos were incubated for 30 min, with gentle swirling every 10 min. Embryos were then incubated at 28.5°C until they were anesthetized in 3-aminobenzoic acid ethyl ester, fixed in 4% paraformaldehyde and processed for in situ hybridization.

DAPT treatments

Wild-type embryos were raised in embryo medium until specified times when DAPT (*N*-[*N*-(3,5-Difluorophenacetyl-L-alanyl]-*S*-phenylglycine-*t*-butyl ester; Calbiochem, La Jolla, California, USA) treatments began. DAPT treatments were performed as previously described (Geling et al., 2002). DAPT was reconstituted with DMSO to make a stock concentration of 10 mM. For experiments, aliquots were diluted to 100 μM in embryo medium. Embryos were dechorionated with watchmakers forceps and placed in the DAPT solution at specified times and incubated overnight at 28.5°C. Control embryos were incubated in an equivalent concentration (1%) of DMSO.

Labeling methods and photomicroscopy

BrdU labeling experiments and detection were performed as described previously (Park and Appel, 2003). BrdU incorporation was detected by immunohistochemistry using anti-BrdU antibody (G3G4, 1:1000, Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA). Rabbit anti-phospho-histone-H3 antibody (1:1000, Upstate Biotechnology, Charlottesville, Virginia, USA) was also used for detection of proliferating cells. For fluorescent detection of antibody labeling, we used Alexa Fluor 568 goat anti-mouse conjugate (1:200, Molecular Probes, Eugene, Oregon, USA). Hoechst labeling was performed by incubating sections in Hoechst stain at 1:1000 dilution for 20 min followed by three washes with PBS. For analysis of floor plate proliferation, control and DAPT-treated embryos were labeled with BrdU. Embryos were sectioned, and immunohistochemistry and Hoechst staining were performed. Each section was observed using epifluorescence optics for the presence of a medial floor plate nucleus labeled by Hoechst stain and the presence or absence of a BrdU-labeled floor plate nucleus. The total number of floor plate nuclei was tabulated, as was the subset that were $BrdU^+$.

Previously described RNA probes include those for *alpha-collagen2a1* (Yan et al., 1995), *twhh* (Ekker et al., 1995), *gdf6a* (Rissi et al., 1995), *gata6* (Ober et al., 2003), *flk1* (Thompson et al., 1998), *ntl* (Schulte-Merker et al., 1994a), and *her4* (Takke et al., 1999). In situ RNA hybridization was performed as described previously (Hauptmann and Gerster, 2000). Embryos for sectioning were embedded in 1.5% agar/30% sucrose and frozen in 2-methyl-butane chilled by liquid nitrogen. 10 μm sections were obtained using a cryostat microtome.

Whole-mount embryos were cleared in methanol and mounted in 75% glycerol. In situ hybridization and Hoechst/immunofluorescence images were obtained using a Retiga Exi cooled CCD camera (Qimaging) mounted on a compound microscope with epifluorescent optics. All images were imported into Adobe Photoshop. Image manipulation was limited to levels, curves, hue and saturation adjustments.

Cell labeling

Fate mapping experiments were performed as described previously (Latimer et al., 2002). For cell fate determination in Notch conditional activation experiments, embryos were obtained from crosses between homozygous *Tg(hsp70l:GAL4)1.5^{kca4}* and hemizygous *Tg(UAS:myc-notch1a-intra)^{kca3}* transgenic lines and injected with 2% caged fluorescein dextran (Molecular Probes) at the 1–2 cell stage. Just before shield stage, these embryos were heat-shocked and then immediately prepared for cell labeling, which was carried out as described previously (Latimer et al., 2002). The embryos were raised until they were 24 hpf, at which time we used morphological criteria to determine those embryos that carried and expressed the activated Notch transgene. These embryos and control embryos were processed for in situ hybridization to mark floor plate and hypochord expression of *col2a1*. To convert photoactivated fluorescein to a blue precipitate, embryos were incubated with alkaline-phosphatase-conjugated anti-fluorescein antibody (Roche Diagnostics) at 1:5000 dilution followed by staining with BCIP/NBT.

Results

The zebrafish shield has distinct midline precursor domains

Our previous fate mapping and gene expression analysis showed that hypochord arises from the lateral edges of the