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Stochasticity and stereotypy in the *Ciona* notochord

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ABSTRACT

Fate mapping with single cell resolution has typically been confined to embryos with completely stereotyped development. The lineages giving rise to the 40 cells of the *Ciona* notochord are invariant, but the intercalation of those cells into a single-file column is not. Here we use genetic labeling methods to fate map the *Ciona* notochord with both high resolution and large sample sizes. We find that the ordering of notochord cells into a single column is not random, but instead shows a distinctive signature characteristic of mediolaterally-biased intercalation. We find that patterns of cell intercalation in the notochord are somewhat stochastic but far more stereotyped than previously believed. Cell behaviors vary by lineage, with the secondary notochord lineage being much more constrained than the primary lineage. Within the primary lineage, patterns of intercalation reflect the geometry of the intercalating tissue. We identify the latest point at which notochord morphogenesis is largely stereotyped, which is shortly before the onset of mediolateral intercalation and immediately after the final cell divisions in the primary lineage. These divisions are consistently oriented along the AP axis. Our results indicate that the interplay between stereotyped and stochastic cell behaviors in morphogenesis can only be assessed by fate mapping experiments that have both cellular resolution and large sample sizes.

Introduction

A fundamental question in developmental biology is the degree to which embryogenesis is stereotyped versus stochastic. Fate mapping experiments in diverse organisms reveal many stereotyped aspects of embryonic development. In the nematode *Caenorhabditis elegans*, the cell divisions and morphogenetic movements of embryogenesis are essentially invariant, and the embryo is small and simple enough that these lineages and movements have been completely described (Sulston et al., 1983). In vertebrates and many other species, however, embryonic development generates predictable patterns but is not invariant from embryo to embryo. This is indicated by the non-deterministic nature of many fate maps (Clarke and Tickle, 1999; England and Adams, 2007), the partially stochastic nature of many developmental processes (Eldar and Elowitz, 2010; Raj and van Oudenaarden, 2008) and the remarkable regulative and plastic properties of some of these embryos (Davidson, 1990; Martinez Arias et al., 2013).

Fate mapping with single cell resolution is not straightforward in vertebrate embryos and other embryos with large cell numbers, so it is possible that their cell lineages and movements may be more stereotyped than currently believed, or that hidden patterns may emerge from finer scale analysis. Embryos with completely stereotyped development can be represented with simple and absolute fate maps. In the more common situation that particular blastomeres may have multiple potential outcomes, either in terms of the cell types they differentiate into or their eventual location within the embryo, then fate maps should ideally be thought of in a probabilistic framework. Single cell labeling by microinjection is a traditional method for fate mapping with single cell resolution, but is technically demanding and difficult to scale up to large sample sizes. It also does not necessarily provide information on lineage relationships within labeled clones. New imaging technologies such as SPIM (Huisken et al., 2004; Keller et al., 2008) raise the possibility of fate mapping entire embryos by in toto time-lapse imaging (Hockendorf et al., 2012; Khairy and Keller, 2010; Megason and Fraser, 2003). Many embryos would need to be imaged, however, to get statistical power on the embryo to embryo variability. Genetic labeling methods offer interesting possibilities for fate mapping with large numbers of replicates (Legue and Joyner, 2010; Livet et al., 2007; Loulier et al., 2014; Salipante and Horwitz, 2007; Yochem and Herman, 2003).

Ascidians are close chordate relatives of the vertebrates and have a conserved chordate embryonic body plan with a particularly small, simple embryo (Munro et al., 2006; Passamaneck and Di Gregorio, 2005). The early lineages in ascidian embryos are invariant and have been described with single cell resolution up to the onset of gastrulation (Nishida, 1987; Nishida and Satoh, 1983, 1985). While many aspects of ascidian morphogenesis are known to be invariant, there are several processes that are at least partly

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stochastic. Foremost among these is the intercalation of the 40 notochord cells into a single-file column. This intercalation process involves mediolaterally-biased intercalation and boundary capture phenomena similar to those observed in vertebrate embryos (Jiang et al., 2005; Munro and Odell, 2002a, 2002b; Veeman et al., 2008). A variety of labeling strategies have shown that the notochord cells from the left and right sides of the embryo intercalate with one another in a stochastic fashion where they do not alternate perfectly (Nishida, 1987; Nishida and Satoh, 1983, 1985). The anterior 32 'primary' notochord cells are derived from blastomeres A7.3 and A7.7, whereas the posterior 8 'secondary' notochord cells are derived from B8.6. Fate mapping experiments in the ascidian *Halocynthia* have suggested that the A7.3 and A7.7 blastomeres that give rise to the anterior 32 notochord cells both contribute randomly to the primary notochord (Nishida, 1987). These observations implied that ascidian notochord intercalation is highly stochastic. In a recent study of how the notochord develops its characteristic tapered shape, we found that certain cell divisions in the notochord primordium are asymmetric such that anterior daughters are smaller than posterior daughters in the anterior of the primordium, whereas posterior daughters are smaller in the posterior of the primordium (Veeman and Smith, 2013). This provided an essential component to our quantitative model of how the notochord becomes tapered, but it implied that there must be a relatively tight mapping between cell position in the early notochord primordium and the intercalated notochord. This challenged the widespread view that ascidian notochord intercalation is highly stochastic. To reconcile these observations, we developed a fine fate map of the *Ciona* notochord. We took advantage of the ability to easily introduce transgenes into the fertilized egg by electroporation (Corbo et al., 1997). This transient transgenesis gives rise to mosaic expression. By varying the amount of DNA used, one can control the degree of mosaicism. It is not clear if the introduced DNA is being propagated as an extrachromosomal array, free plasmid or some other fashion, but there is good evidence that the mosaic expression is clonal in nature (Corbo et al., 1997; Zeller et al., 2006). Here we deliberately used low doses of a tissue-specific GFP reporter plasmid to label small clones of cells in the notochord. The advantage of this method is that very large numbers of clones can be generated as compared to traditional fate mapping by single blastomere microinjection. The blastomere that was labeled to give rise to any particular clone is not known, but the large numbers possible allow distinct classes of clone to be identified and retrospectively associated with particular blastomeres.

Methods

Embryo culture and electroporation

Adult *Ciona intestinalis* were obtained from Marine Research and Educational Products (San Diego, CA) and housed in a recirculating aquarium. Fertilized eggs were dechorionated by standard methods and electroporated with a Bio-Rad Gene Pulser Xcell using Time Constant mode with settings of 50 V, time constant 17 ms and a 4mm electrode gap. The electroporation solution was made by adding 300 μl of dechorionated eggs in artificial seawater to a mixture of 480 μl 0.77 M mannitol and 20 μl of DNA. Typical experiments used only 5–10 μg of a plasmid driving GFP expression under the control of the notochord-specific *Brachyury* promoter/enhancer (*Bra* > GFP). Electroporated eggs were moved to dishes of artificial seawater containing 0.01% BSA and cultured at 21 °C until the desired stage. Embryos were staged according to Hotta's staging series (Hotta et al., 2007).

Cell lineage nomenclature

We use the standard Conklin (1905) system for naming blastomeres, following Meinertzhagen's convention for later blastomeres whereby the more anterior blastomere receives the higher (even) number (Cole and Meinertzhagen, 2004). The primary notochord is derived from the left and right side equivalents of blastomeres A7.3 and A7.7. In this nomenclature system the first letter encodes the parental blastomere at the 8-cell stage (A vs. P and animal vs. vegetal), the first number indicates the cell cycle generation and the third number indicates the blastomere number. A7.3 for example divides to give A8.6 and A8.5. A8.6 divides to give rise to A9.12 and A9.11 …. The postmitotic primary notochord consists of A10.17–A10.24 and A10.49–A10.56. Right side blastomeres are indicated with an asterisk.

Staining and imaging

Embryos were fixed overnight in 2% paraformaldehyde in artificial seawater then washed several times with PBS + 0.2% Triton X-100 (PBSTr). Blocking and labeling steps used PBSTr with 5% heat inactivated goat serum. Embryos were labeled using a polyclonal antibody against GFP and Alexa-labeled secondary antibodies (Invitrogen). We also used Bodipy-FL-phallacidin (Invitrogen) to label cell cortices. Stained embryos were adhered to poly-L-lysine coated coverslips, dehydrated through a brief isopropanol series, cleared and mounted in Murrays Clear (BABB), and imaged on a Zeiss 700 laser scanning confocal microscope, typically using a 40× 1.3NA objective.

Analysis

Confocal stacks were opened using FIJI/ImageJ (Schindelin et al., 2012; Schneider et al., 2012) and carefully examined to ensure that the embryos showed no obvious defects in development. Each notochord cell from front to back was scored as being either GFP expressing or not expressing. We only considered embryos with 16, 8 or 4 labeled cells in the anterior 32 primary notochord cells, and/or 4 labelled cells in the posterior 8 secondary notochord cells. We only rarely observed evidence of any mixing between the primary and secondary lineages. These embryos usually had defects in morphogenesis and were not considered further. We frequently found that labeled populations of cells could be further resolved based on the intensity of expression, e.g. embryos with 8 labeled cells often had 4 strongly expressing cells and 4 weakly expressing cells. This likely reflects asymmetric segregation of the electroporated plasmid. In that case, we would have scored it as both an 8-cell event and two 4-cell events. We also identified embryos with 16 labeled cells with 8 expressing strongly and 8 weakly. Embryos with more complex combinations of labeled cells were also common but not included in our analyses. All statistical analyses and simulations were performed in Matlab (Mathworks).

Results and discussion

Ciona transgenesis by electroporation is known to give rise to mosaic expression in a clonal fashion (Corbo et al., 1997; Zeller et al., 2006). In the course of other experiments, we noted that it was relatively common to find embryos electroporated with notochord-specific expression constructs that had exactly 16, 8 or 4 expressing cells in the primary notochord. More complex expression patterns predominated when high doses of plasmid were electroporated, likely reflecting the superposition of multiple clonal events. When electroporating low doses of plasmid, however, then embryos with