Developmental Biology 394 (2014) 170–180

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Evolution of Developmental Control Mechanisms

Ephrin-mediated restriction of ERK1/2 activity delimits the number of pigment cells in the *Ciona* CNS

Nicolas Haupaix [a,b,c,1], Philip B. Abitua [d,1], Cathy Sirour [a,b], Hitoyoshi Yasuo [a,b], Michael Levine [d], Clare Hudson [a,b,*]

[a] CNRS, Laboratoire de Biologie du Développement de Villefranche-sur-mer, Observatoire Océanologique, 06230 Villefranche-sur-mer, France
[b] Sorbonne Universités, UPMC Univ Paris 06, Laboratoire de Biologie du Développement de Villefranche-sur-mer, Observatoire Océanologique, 06230 Villefranche-sur-mer, France
[c] Université de Nice Sophia Antipolis, Nice, France
[d] Center for Integrative Genomics, Division of Genetics, Genomics and Development, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA

ARTICLE INFO

Article history:
Received 20 March 2014
Received in revised form
4 July 2014
Accepted 10 July 2014
Available online 22 July 2014

Keywords:
Melanocyte
Pigment cell
Ascidian
Neural crest
Oriented cell division

ABSTRACT

Recent evidence suggests that ascidian pigment cells are related to neural crest-derived melanocytes of vertebrates. Using live-imaging, we determine a revised cell lineage of the pigment cells in *Ciona intestinalis* embryos. The neural precursors undergo successive rounds of anterior–posterior (A–P) oriented cell divisions, starting at the blastula 64-cell stage. A previously unrecognized fourth A–P oriented cell division in the pigment cell lineage leads to the generation of the post-mitotic pigment cell precursors. We provide evidence that MEK/ERK signals are required for pigment cell specification until approximately 30 min after the final cell division has taken place. Following each of the four A–P oriented cell divisions, ERK1/2 is differentially activated in the posterior sister cells, into which the pigment cell lineage segregates. Eph/ephrin signals are critical during the third A–P oriented cell division to spatially restrict ERK1/2 activation to the posterior daughter cell. Targeted inhibition of Eph/ephrin signals results in, at neurula stages, anterior expansion of both ERK1/2 activation and a pigment cell lineage marker and subsequently, at larval stages, supernumerary pigment cells. We discuss the implications of these findings with respect to the evolution of the vertebrate neural crest.

Introduction

The ascidian pigment cells form part of the ocellus and the otolith, light and gravity sensing organs, respectively (Jiang et al., 2005; Tsuda et al., 2003). Previously, the ascidian pigment cells have been proposed as the evolutionary precursors of the vertebrate retinal pigment epithelium or pineal organ, based on expression of gene markers and the probable function of the ocellus pigment cell in shielding the photoreceptors (Lamb et al., 2007; Sato and Yamamoto, 2001). However, recent evidence using a larger panel of genes supports a closer evolutionary relationship to the cephalic neural crest of vertebrates (Abitua et al., 2012; Ivashkin and Adameyko, 2013). This hypothesis is strengthened by the acquisition of migratory properties upon the misexpression of Twist, a cell behavior exhibited by neural crest.

Of the four founder embryonic lineages of *Ciona* (A- and B- of the vegetal hemisphere and a- and b- of the animal hemisphere), the central nervous system (CNS) derives from three lineages, A-, a- and b- (Nishida, 1987). This study is concerned with the a-line neural lineages, from which the pigment cells arise. At the 64- to 76-cell stage, the a-line neural lineages form one row of six cells (Fig. 1) (Lemaire et al., 2002). These cells then divide twice along the A-P axis to generate four rows of six cells at the 6-row neural plate stage (Fig. 1). The 6-row neural plate is arranged with row I posterior-most and row VI anterior-most (Nicol and Meinertzhagen, 1988). The a-line cells comprise rows III–VI (pink cells in Fig. 1) and the A-line cells, rows I and II. Of the a-line cells, only rows III and IV will undergo neurulation and contribute to the CNS. These two rows generate the anterior part of the sensory vesicle, which is the ascidian larval brain, and also contribute to the oral siphon primordium (Christiaen et al., 2007; Cole and Meinertzhagen, 2004; Nishida, 1987; Taniguchi and Nishida, 2004; Veeman et al., 2010). The anterior two rows, V and VI, generate the palps, an adhesive organ, as well as non-neural ectoderm. The pigment cell lineage is situated in the lateral-most cell of row III. Between the 6-row neural plate and the 12-row neurula stages,

* Corresponding author at: CNRS, Laboratoire de Biologie du Développement de Villefranche-sur-mer, Observatoire Océanologique, 06230 Villefranche-sur-mer, France. Fax: +33 493763982.
E-mail address: hudson@obs-vlfr.fr (C. Hudson).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.ydbio.2014.07.010
0012-1606/

each row of cells divides in a specific sequence, with row II cells dividing first, followed by row I, then row III and V and lastly row IV and VI (Nicol and Meinertzhagen, 1988). In each row, cells in the medial four columns divide prior to those in the lateral columns. At the 12-row neural plate stage, the pigment cell lineage is segregated into the posterior row III (IIIp) cell, a10.97. As we will describe in this manuscript, the a10.97 pigment cell precursors undergo a further and final round of cell division along the A-P axis, whereby the posterior-positioned daughters, a11.193, intercalate at the midline to become the pigment cells of the otolith and ocellus.

FGFs are secreted ligands that bind to a class of receptor tyrosine kinases, the FGF receptors (FGFRs), and predominantly activate the Ras/MEK/ERK cascade. FGF/ERK signaling is implicated in a vast range of processes during vertebrate neural development including the induction of neural crest (Guillemot and Zimmer, 2011; Hébert, 2011; Stuhlmiller and Garcia-Castro, 2012). During ascidian neural development, FGF/ERK signaling is responsible for the specification of different cell types. It is involved in the process of neural induction of a-line neural lineages at the 32-cell stage (Bertrand et al., 2003) and later during patterning of the developing neural plate and neural tube (Abitua et al., 2012; Hudson et al., 2003, 2007; Squarzoni et al., 2011; Stolfi et al., 2011; Wagner and Levine, 2012). Recently, Eph/ephrin signaling via RasGAP has been shown to limit the spatial extent of activation of ERK1/2 during several developmental cell fate choices in *Ciona* embryos including during neural development (Haupaix et al., 2013; Ohta and Satou, 2013; Picco et al., 2007; Shi and Levine, 2008; Stolfi et al., 2011). Here we investigated the role of Eph/ephrin signaling and the MEK/ERK pathway during pigment cell specification at neural plate stages of *Ciona intestinalis* development.

Materials and methods

Embryo experiments and constructs

Adult *C.intestinalis* were purchased from the Station Biologique de Roscoff (France) or M-Rep (San Diego, CA). Cell nomenclature, lineage and the fate maps are previously described (Cole and Meinertzhagen, 2004; Conklin, 1905; Nishida, 1987). Ascidian embryo culture has been described (Sardet et al., 2011). Electroporation was carried out as described (Christiaen et al., 2009). U0126 (Calbiochem) was added to artificial seawater at 2 μM. All data was pooled from at least two independent experiments.

For the electroporation constructs, the upstream sequences *ZicL*, *Tyr*, *Mitf*, *Dmrt*, *Msx* and *Fog* are previously described (Abitua et al., 2012; Haupaix et al., 2013; Rothbächer et al., 2007; Wagner and Levine, 2012). These were used to drive expression of *LacZ*, *mCherry*, *Eph1ΔC*, *Histone 2B* (*H2B*), *unc76GFP*, *Eph3ΔC*, *RG*(*R818E*) (Abitua et al., 2012; Haupaix et al., 2013; Hudson et al., 2007; Stolfi et al., 2011; Wagner and Levine, 2012). Following electroporation, embryos were selected at gastrula stages for correct morphology. In Fig. 3, *Dmrt::Eph3ΔC* electroporated embryos were co-electroporated with *Trp::mCherry*. Only fluorescent (i.e. electroporated) embryos were counted for this experiment, in all other experiments, all embryos were counted. In order to assess ocellus and otolith pigment cell type, embryos were mounted in 80% glycerol and slightly compressed. Ocellus type or otolith type pigment cells were defined based on pigment granule morphology; such that tiny dispersed granules were defined as ocellus type and larger, round or flower shaped granules were defined as otolith. The number of ocellus type pigment cells was likely underestimated since it was more difficult to distinguish individual ocelli from one another. In both control and electroporated embryos, even very large ocelli were counted as one.

In situ hybridization and immunofluorescence

In situ hybridization was carried out as previously described (Hudson et al., 2013). Dig-labeled probes were synthesized from the following *Ciona* cDNA clones: *Trp* (Hudson et al., 2003), *FoxC* (cilv050a24) (Imai et al., 2004), *ZicL* (cicl002e04) (Imai et al., 2002b), *Mitf* (cilv41b12) (Abitua et al., 2012). *Six3/6* (cicl021e08), *Dll-B* (cicl022f04), *ephrinAc* (ciad074h16), *ephrinAb* (cieg037l08), *ephrinAd* (ciad008n17), *Eph3* (cieg009e01), *FgfR* (citb040h06), *Fgf9/16/20* (citb007k01) and *Fgf8/17/18* (citb002j04) are all described (Imai et al., 2004). Clone numbers refer to clones from the 'Release 1 Gene Collection Plates' (Satou et al., 2002). For *Dll-B* in situ hybridization, DAPI staining was used to verify cell identity. In some batches of embryos, *Dll-B* was also detected in row IV. These batches of embryos were removed from the analysis. For *Trp* in situ hybridization in Fig. 5, it should be noted that the intensity of the *Trp* signal increases with developmental time. At the 6-row neural plate stage, the color reaction was developed for approximately 12 h, whereas at the early tailbud stage, the color reaction time was 1 to 3 h.

dpERK1/2 immunofluorescence is described previously (Haupaix et al., 2013). LacZ immunofluorescence is described previously (Stolfi et al., 2011) except that anti-β-galactosidase (Molecular Probes, A11132) was used at 1/500 and the secondary antibody used was goat anti-rabbit Alexa Fluor 488 (Molecular Probes, A11008) at 1/1000.

In our analysis, we refer to embryos as the 6-row neural plate stage to indicate that no row of a-line cells has divided, though cells in row II have sometimes divided. We refer to the 12-row neurula stage to indicate that row III cells have divided. Embryos in Figs. 2 and 6 and Supplementary Fig. 1 were mounted in VECTASHIELD/DAPI (Vector Laboratories). Embryos in Figs. 2, 5–7 and Supplementary figures were photographed on an Olympus BX51 using Leica DFC310FX camera and those in Figs. 3 and 4G on a Zeiss Axio Imager AZ using a SPOT RT3 camera.

Time-lapse movie

The time-lapse sequence was obtained using a Zeiss LSM 700 microscope using a plan-apochromat 20 × objective. Confocal stacks contained 20 optical slices at a thickness of approximately 1 μm each and were taken every 3 min for 2 h. Images were rendered in 3D using Volocity 6 with the 3D opacity visualization tool.

Results

Differential activation of ERK1/2 in the neural plate is required for patterning along the A–P axis

It has previously been shown that differential ERK1/2 activity between rows III/IV (CNS) and V/VI (palp) at the 3-row neural plate stage (Fig. 1) accounts for acquisition of their distinct genetic programs (Wagner and Levine, 2012). ERK1/2 is subsequently differentially activated between rows III and IV at the 6-row neural plate stage with ERK1/2 active in row III cells (Fig. 2A) (Hudson et al., 2007). This pattern of ERK1/2 activation is required for the differential fate specification of these two sister rows (Fig. 2C). We inhibited MEK/ERK signal transduction by applying U0126, an inhibitor of the MAP kinase kinase, MEK1/2, at precise developmental time points. The following markers were used to assess neural plate patterning: *FoxC* (rows V and VI), *Dll-B* (epidermis and rows V and VI), *ZicL* (rows III and IV), *Six3/6* (row IV), *Mitf* and *Trp* (row III) (Abitua et al., 2012; Irvine et al., 2007; Wagner and Levine, 2012). As reported previously, application of U0126 at the 76-cell stage disrupts the choice between row III/IV and row V/VI fates