Available online at www.sciencedirect.com
ScienceDirect
Developmental Biology 301 (2007) 227–239
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

In vivo analysis reveals a critical role for neuropilin-1 in cranial neural crest cell migration in chick

Rebecca McLennan, Paul M. Kulesa *

Stowers Institute for Medical Research, 1000 E. 50th St., Kansas City, MO 64110, USA

Received for publication 13 April 2006; revised 2 August 2006; accepted 7 August 2006
Available online 10 August 2006

Abstract

The neural crest provides an excellent model system to study invasive cell migration, however it is still unclear how molecular mechanisms direct cells to precise targets in a programmed manner. We investigate the role of a potential guidance factor, neuropilin-1, and use functional knockdown assays, tissue transplantation and in vivo confocal time-lapse imaging to analyze changes in chick cranial neural crest cell migratory patterns. When neuropilin-1 function is knocked down in ovo, neural crest cells fail to fully invade the branchial arches, especially the 2nd branchial arch. Time-lapse imaging shows that neuropilin-1 siRNA transfected neural crest cells stop and collapse filopodia at the 2nd branchial arch entrances, but do not die. This phenotype is cell autonomous. To test the influence of population pressure and local environmental cues in driving neural crest cells to the branchial arches, we isochronically transplanted small subpopulations of DiI-labeled neural crest cells into host embryos ablated of neighboring, premigratory neural crest cells. Time-lapse confocal analysis reveals that the transplanted cells migrate in narrow, directed streams. Interestingly, with the reduction of neuropilin-1 function, neural crest cells still form segmental migratory streams, suggesting that initial neural crest cell migration and invasion of the branchial arches are separable processes.

Keywords: Neuropilin-1; Neural crest; Cranial; Cell migration; Chick; Confocal; Time-lapse imaging

Introduction

The proper assembly of the vertebrate head and peripheral nervous system crucially depends on the emergence and accurate targeting of intrinsically migratory, multipotent cells, called the neural crest. The programmed invasion of the neural crest requires a complex interplay between signals from the neural tube, the surrounding environment and the ability of the cells to properly interpret guidance cues. Defects in any single aspect of this event may translate into craniofacial and cardiac abnormalities and enteric and other autonomic nervous system malformations (Mooney and Siegel, 2002; Farlie et al., 2004; Gershon and Ratcliffe, 2004). Cranial neural crest cells (NCCs) contribute to neurons and glia of the sensory ganglia and to bone, cartilage and pigment cells of the face and neck (Baker and Bronner-Fraser, 1997; Le Douarin and Kalcheim, 1999; Le Douarin et al., 2004). In a striking pattern, cranial NCCs form 3 discrete migratory streams throughout the head, a pattern that is re-capitulated in a wide range of vertebrate systems (Le Douarin and Kalcheim, 1999). The exit points of the streams correlate with specific segmental structures of the hindbrain (Lumsden et al., 1991; Lumsden and Krumlauf, 1996), called rhombomeres. Cranial NCCs from rhombomere 1 (r1), r2 and r3 migrate in a wide stream to populate the 1st branchial arch (Kontges and Lumsden, 1996). NCCs from r3 to r5 sort into a stream that extends lateral to r4 and expands to populate the 2nd branchial arch (ba2). The 3rd branchial arch is composed of NCCs from r5 and r6. Given the complexity of a symphony of cell signaling interactions between the NCCs, the neural tube and the environment, a major question in vertebrate development is how molecular mechanisms consistently produce the stereotypical cranial NCC migratory pattern.

The combination of tissue transplantation studies, cell tracing and time-lapse analyses in a variety of embryonic systems has contributed to portraying the complexity of NCC migratory behaviors (Bard and Hay, 1975; Newgreen et al., 1979; Tucker and Erickson, 1984; Schilling and Kimmel, 1994;

* Corresponding author. Fax: +1 816 926 2074.
E-mail address: pmk@stowers-institute.org (P.M. Kulesa).

0012-1606/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2006.08.019

Kulesa and Fraser, 1998; Halloran and Berndt, 2003; Young et al., 2004). However, it is still unclear how the neural crest interprets intrinsic and environmental signals such that a programmed pattern of invasion emerges. For example, one of the most striking discrete NCC migratory streams extends lateral to r4. Cell labeling studies in chick and mouse show that the cells that emigrate from mid-r3 to mid-r5 contribute to the r4 migratory stream (Sechrist et al., 1993; Birgbauer et al., 1995; Trainor and Krumlauf, 2000). Curiously, the regions adjacent to r3 and r5 remain relatively void of NCCs. It has been suggested that signals within the neural tube may regulate the number of neural crest cells such that r3 and r5 produce fewer NCCs than the even-numbered rhombomeres (Graham et al., 1993, 2004). In contrast, there is strong evidence that environmental signals adjacent to the neural tube play a role in neural crest cell guidance (Trainor and Krumlauf, 2001). Time-lapse studies in chick reveal that a subpopulation of r3 and r5 neural crest cells may migrate into the regions lateral to r3 or r5, but quickly reroute trajectories towards a neighboring stream (Kulesa and Fraser, 1998) and populate the branchial arches. When quail r2 or r4 NCCs are transplanted lateral to r3 in host chick embryos, the cells divert to neighboring streams (Farlie et al., 1999), further suggesting the presence of an inhibitory signal. However, when neural crest cell migratory pathways are blocked by foil barrier transplantation in chick, trailing neural crest cells move around the barriers and re-target towards the branchial arches (Kulesa et al., 2005), suggesting that cell trajectories are not predetermined. Interestingly, chick NCCs can be diverted into the region lateral to r3 when the r3 neuroepithelium and r3 surface ectoderm are ablated (Golding et al., 2002, 2004). Thus, interactions between the emerging NCCs and the local environment play an important role in sculpting and maintaining the discrete NCC migratory streams.

Although several molecular candidates involved in cranial NCC guidance have been identified, the in vivo functional studies are just beginning to emerge (Le Douarin et al., 2004). Previous studies have implicated neuropilin-1 in the migration of both cranial and trunk NCCs from the neural tube to their proper destinations (Eickholt et al., 1999; Osborne et al., 2005). Neuropilin-1, a type 1 membrane protein, is composed of a large extracellular domain and a small cytoplasmic region (reviewed by He et al., 2002; Fujisawa, 2003). Neuropilins act as co-receptors with plexins for secreted forms of semaphorins. Studies suggest that neuropilins bind the corresponding semaphorins and initiate intracellular signaling transduction via plexins (reviewed by Tamagnone and Comoglio, 2000). In 1996, Kawakami and colleagues demonstrated that Neuropilin-1 is expressed by numerous cranial nerves in the murine embryo, including the facial nerve in the 2nd branchial arch (Kawakami et al., 1996). Since then, *Neuropilin-1* has been shown to be expressed in discrete tissues of the chick embryo. Specifically, *Neuropilin-1* is expressed at the transcript level by migrating cranial NCCs (Eickholt et al., 1999; Gammill and Bronner-Fraser, 2002). Cranial NCCs also express *Plexin-A1* transcripts, while *semaphorin 3A* and *semaphorin 3F* transcripts are expressed in odd-numbered rhombomeres (Eickholt et al., 1999; Osborne et al., 2005). Interestingly, chick cranial NCCs avoid substrates containing semaphorin 3A in vitro (Eickholt et al., 1999). These experiments suggest that semaphorin–neuropilin interactions play a role in the initial sculpting cranial NCC streams.

In this paper, we investigate an in vivo role for neuropilin-1 signaling in cranial NCC guidance. To address the in vivo function of neuropilin-1, we use a loss-of-function approach and take advantage of a neuropilin-1 siRNA/EGFP construct (Np-1 siRNA) (Bron et al., 2004) and neuropilin-1–Fc (Np-1–Fc) to perturb endogenous neuropilin-1–ligand interactions. We compare the in vivo migratory patterns of fluorescently labeled Np-1 siRNA transfected and untransfected DiI-labeled cranial NCCs in chick, using 3D confocal microscopy. We analyze the effects of a reduction in neuropilin-1 function on NCC trajectories and cell morphologies using time-lapse confocal imaging. To test the influence of potential inhibitory and permissive cues lateral to the neural tube, we transplant small subgroups of DiI-labeled NCCs isochronically into host embryos ablated of premigratory neighboring neural crest and analyze transplanted cell trajectories over time. We propose a model in which neuropilin-1 is required for cranial NCCs to properly invade specific branchial arches and suggest that multiple, distinct mechanisms shape neural crest cell migratory streams and branchial arch invasion.

Materials and methods

Embryos

Fertilized white leghorn chicken eggs (supplied by Ozark Hatchery, Oeosho, MO, USA) were incubated at 38°C in a humidified incubator until the desired stages of development. Eggs were then rinsed with 70% ethanol and 3 ml of albumin was removed from under the yolk with a 5 ml syringe (309603, Becton Dickinson, Franklin Lakes, NJ, USA) and an 18 gauge needle (305196, Becton Dickinson). A window was cut into the shell and the embryos were visualized by injecting 10% India ink (Pelikan Fount; PLK 51822A143, www.mrart.com, Houston, TX, USA) underneath the area opaca and area pellucida with a 1 ml syringe (309628, Becton Dickinson) and a 25 gauge needle (305112, Becton Dickinson).

Immunohistochemistry

Immunostaining with neuropilin-1 (a kind gift from the Fujisawa laboratory) and HNK-1 was performed on both vibratome sections and whole mount embryos. Both neuropilin-1 (1:100) and HNK-1 (1:20), diluted in 10% goat serum, 4% bovine serum albumen, 0.1% triton-X-100 in phosphate buffered saline (PBS), were incubated with whole mount embryos, hindbrain half preparations or 150 μm vibratome sections overnight at 4°C. After repeated washes, secondary antibodies were applied and incubated at 4°C overnight. The secondary antibodies used for neuropilin-1 and HNK-1 were Alexa Fluor goat anti-rabbit IgG 488 (A-11008, Molecular Probes, Eugene, OR, USA) and Alexa Fluor goat anti-mouse IgM 546 (A-21045, Molecular Probes), respectively.

In ovo electroporation and cell labeling

Embryos were incubated until Stage 9 (Hamburger and Hamilton, 1951), when 6–8 somite pairs were visible. Once an embryo was accessed and visualized, a few drops of sterile Ringer's solution were added over the embryo to prevent it from drying out. A small area of the vitelline membrane above the hindbrain region was removed with a sharpened tungsten needle. Plasmid DNA (5 μg/μl) was injected into the lumen of the neural tube at the axial level of the rostral hindbrain using a pulled borosilicate glass needle (BF100-50-10, Sutter,