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SCIENCE DIRECT
Developmental Biology 287 (2005) 125 – 133
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

The pattern of neural crest advance in the cecum and colon ☆

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Received for publication 9 April 2005, revised 19 August 2005, accepted 22 August 2005
Available online 28 September 2005

Abstract

Neural crest cells leave the hindbrain, enter the gut mesenchyme at the pharynx, and migrate as strands of cells to the terminal bowel to form the enteric nervous system. We generated embryos containing fluorescent enteric neural crest-derived cells (ENCCs) by mating Wnt1-Cre mice with Rosa-floxed-YFP mice and investigated ENCC behavior in the intact gut of mouse embryos using time-lapse fluorescent microscopy. With respect to the entire gut, we have found that ENCCs in the cecum and proximal colon behave uniquely. ENCCs migrating caudally through either the ileum, or caudal colon, are gradually advancing populations of strands displaying largely unpredictable local trajectories. However, in the cecum, advancing ENCCs pause for approximately 12 h, and then display an invariable pattern of migration to distinct regions of the cecum and proximal colon. In addition, while most ENCCs migrating through other regions of the gut remain interconnected as strands; ENCCs initially migrating through the cecum and proximal colon fragment from the main population and advance as isolated single cells. These cells aggregate into groups isolated from the main network, and eventually extend strands themselves to reestablish a network in the mid-colon. As the advancing network of ENCCs reaches the terminal bowel, strands of sacral crest cells extend, and intersect with vagal crest to bridge the small space between. We found a relationship between ENCC number, interaction, and migratory behavior by utilizing endogenously isolated strands and by making cuts along the ENCC wavefront. Depending on the number of cells, the ENCCs aggregated, proliferated, and extended strands to advance the wavefront. Our results show that interactions between ENCCs are important for regulating behaviors necessary for their advancement.

Keywords: Enteric nervous system; Neural development; YFP; Wnt1; Cre recombinase; Neural crest; Strand migration; Cecum; Population pressure; GDNF; Endothelin

Introduction

Neural crest cells leave the hindbrain, enter the foregut, and advance along the entire length of the gastrointestinal tract as enteric neural crest-derived cells (ENCCs) by a combination of migration and proliferation. During this period of migration and proliferation, some of these ENCCs are differentiating into neurons. Coordination of these activities results in the colonization of the gut and establishment of ganglia containing the neurons and glia that constitute the enteric nervous system. Proliferation plays an important role and is required to maintain a population of migrating crest to offset the number of crest cells that differentiate as colonization progresses. This is underscored by the fact that the migratory path of ENCCs is longer than that that of other crest-derived cells. If the number of crest cells entering the gut is substantially reduced, they do not reach the terminal bowel (Burns et al., 2000; Peters-van der Sanden et al., 1993). These and other data (Young et al., 2004b) indicate a relationship between the number of cells and the extent of migration although this relationship has not been examined at the cellular level. In addition, a number of ligands and their receptors play critical roles in influencing movement and maintaining proliferation of enteric crest cells (Young et al., 2004a) although their role in regulating the migration of ENCCs in situ is not clear.

Migration of the ENCCs is essential for the distribution of the cells throughout the gut. In fixed preparations of developing gut, ENCCs are arranged largely in strands or cords of cells (Epstein et al., 1991; Young et al., 1998, 1999; Conner et al., 2003). The most caudal cells of the strands represent the migratory wavefront, while rostral to the wavefront, the crest cells show a pattern of branches and nodes that presages the organization of the adult ENS. Strands of colonizing crest are not limited to the gut; cardiac crest cells also appear in cords or chains as they move into the heart (Poelmann et al., 1998). In living preparations, strands of

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0012-1606/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2005.08.040

crest cells are also seen in branchial arches (Kulesa and Fraser, 2000; Teddy and Kulesa, 2004) and during the formation of peripheral ganglia (Kasemeier-Kulesa et al., 2005). This pattern of strand migration also occurs in the CNS where the rostral migratory stream advances into the olfactory bulb (Lois et al., 1996), and neuroblasts move through the subcortex (Luzzati et al., 2003; Imitola et al., 2004). In addition to neural precursors, endothelial cells advance as strands to form vessels in the developing brain (Noden, 1991). Migration in the form of cellular chains may be more widespread than initially appreciated.

Direct observation of ENCC migration in situ was first studied using mice in which cDNA encoding tau-EGFP-myc had been inserted into the first exon of the Ret gene (Young et al., 2004b). These mice expressed GFP in all ENCCs, and permitted the study of ENCC movement using time-lapse imaging. Their results revealed that ENCCs migrate as chains, and that the leading cells of strands follow complex and unpredictable trajectories. Although their studies describe the movement of ENCC in the ileum and colon, they were unable to visualize the movement of ENCC in the cecum and proximal colon because of its complex geometry and in the distal colon because of the weak fluorescence intensity of the ENCCs. We have used a different transgenic mouse, one in which YFP is constitutively expressed after activation by the Wnt-1 promoter, to study the dynamics of ENCCs in the cecum, proximal, and distal colon. Images taken at multiple planes together with a greater ENCC fluorescent signal from this preparation permitted an analysis of ENCC dynamics in these regions. Our study shows that the ENCC wavefront regularly pauses at the cecum and then displays a very different pattern of migration from that found in other more proximal regions. Instead of advancing as a network of strands, ENCCs migrate into the cecum and proximal colon initially as isolated single cells. These isolated cells form groups, which over time extend strands towards other isolated groups of cells. This interaction reestablishes an unbroken network that is capable of advancing towards the terminal colon. In the terminal colon, sacral crest cells extend a short distance rostrally and join the vagal crest population. Lastly, we show a relationship between ENCC number, interaction, and migratory behavior by analyzing naturally and artificially isolated strands in the colon at the wavefront. Therefore, in addition to showing ENCC behavioral changes in the cecal region, our results show a hitherto unidentified response to decreased cell number; that is, aggregation, proliferation, and then strand elongation.

Materials and methods

Animals

Transgenic mice expressing Cre recombinase under the control of the Wnt1 promoter/enhancer (Danielian et al., 1998) were mated to mice containing either floxed GFP-R26 (Mao et al., 2001) or YFP-R26R (Srinivas et al., 2001). Male mice heterozygous for the Wnt1-Cre (Wnt−/+) were mated to females homozygous for floxed GFP (R26−/−) or YFP (R26R−/−). Mice were genotyped by PCR using primers to Cre recombinase (Cre F; 5′-CTGGTGTAGCTGATGATCCG-3′, Cre R; 5′-ATGGCTAATCGCCATCTTCC-3′), YFP-R26R (Soriano, 1999), and GFP-R26 (Zambrowicz et al., 1997). Pregnant mice from timed mating were killed by cervical dislocation. The University of Wisconsin Animal Care Committee approved these procedures. The day of the vaginal plug was considered embryonic day (E) 0.5. Embryos were removed from the uterus between E10.5 and 13.5, staged, and time-lapse studies were initiated immediately.

Time-lapse microscopy

The gastrointestinal tract from YFP+ or GFP+ embryos was suspended across the hole punched into a piece of a GS Millipore filter (Hearn et al., 1999) secured by silicone grease, to a Wilco glass bottom dish (W-P Instruments, FL). The culture dish was filled with DMEM/F12 media (2 mM glutamine, 0.075% sodium bicarbonate) containing 2% B-27 supplement (Gibco, Grand Island, NY), 100 u/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (Gibco). Media were covered with a layer of mineral oil to prevent evaporation (heavy paraffin oil; Fisher Scientific, Pittsburg, PA). The dish was placed on a heated stage (Fryer Co, Huntley, IL) of an inverted stage microscope (Nikon Diavert) equipped with a Uniblitz shutter (Vincent Associates, Rochester, NY), a CCD camera (Cool Snap, Roper Scientific, Tucson, AZ), and a motorized stage driver (Prior Instruments, Rockland, MA). Images were taken with a 10× objective every 7–14 min, after 20–40 μs exposures, at 6–9 focal planes separated by a Z-distance of 10–35 μm. The cells did not show any evidence of phototoxicity such as membrane blebbing.

Neural crest cells and some central nervous system cells in the developing neural tube express Wnt-1 transiently (Ikeya et al., 1997; Molven et al., 1991; Wilkinson et al., 1987). Although the Wnt-1/Cre transgene has been found in some neural crest derivatives (Chai et al., 2000; Jiang et al., 2000; Kapur, 2000), we have extended this list and confirmed that $Wnt^{-/+}$/$R26R^{-/+}$ embryos contained fluorescent cells in the neural crest-derived tissues including cranial mesenchyme, dorsal root ganglia, and sympathetic ganglia (not shown). Most experiments used YFP+ embryos, which showed a greater intensity of fluorescence than the GFP+ embryos. All YFP+ cells were also positive for p75 indicating that all the YFP+ cells are ENCC (not shown). The overall intensity of ENCC fluorescence did not vary between E10.5 and E13.5, but individual cells showed slight differences in YFP intensity in all regions of the gut.

Cell tracking

Images were processed and analyzed with digital imaging software (Metamorph, Universal Imaging. West Chester, PA). The speed of wavefront advance was taken from preparations filmed at least 12 h, and determined by the difference between the position of the most distal cell on a strand over 6 h intervals. The rates of movement of single cells observed either caudal to, behind, or within strands, were taken from preparations filmed at least 6 h. Single cell rates were determined by taking the average of the distance each cell moved over 7–14 min intervals for 2–14 h.

Tissue preparation

YFP+ hindgut from E11.5, E12.5, and E13.5, was cut using a tungsten needle at various sites with respect to the wavefront. Segments of gut caudal to the cut were then affixed to GS Millipore filter paper as described above. The gut was either cultured in an incubator or in the chamber as described above for concurrent time-lapse recording. Images ENCCs caudal to the cut were taken at 15–20 min intervals. Analysis of wavefront and single cell movement from these preparations is as described above.

Immunohistochemistry

After filming, the gut was removed, fixed in 4% paraformaldehyde for 1 h, permeablized in Triton X-100, washed, and immunostained with antibodies to p75 (Promega, Madison, WI), and goat anti-rabbit conjugated to Texas Red (Jackson Immunochemicals, West Grove, PA).

Results

ENCC behavior changes in the cecum

ENCCs predominantly colonize the gut in the form of strands of connected cells. Most often, these strands appear to