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Developmental Biology 287 (2005) 125 – 133
DEVELOPMENTAL BIOLOGY
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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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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

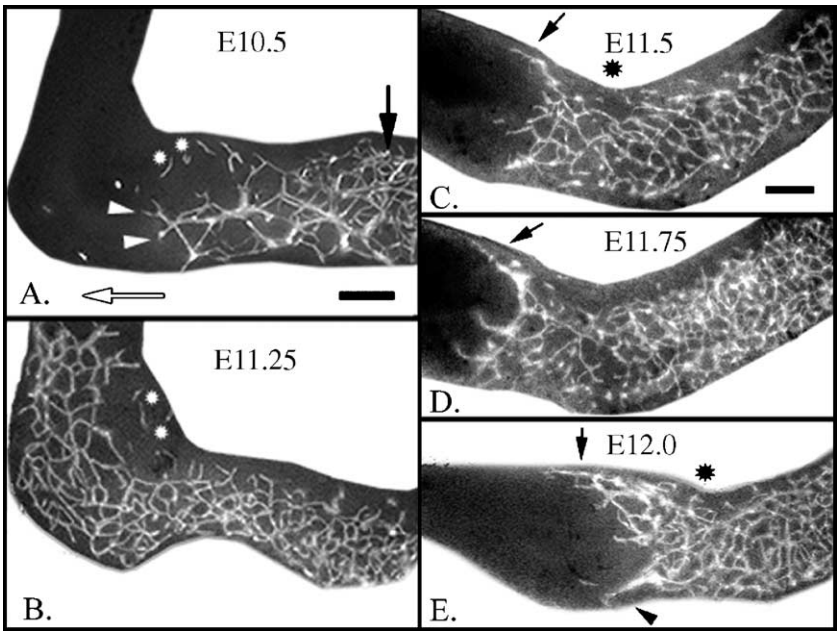

Fig. 1. From E10.5 to E11.25, ENCCs progress through the ileum. At E10.5 (A), strands extend caudally into the uncolonized ileum (arrowheads). A few cells inhabit the mesentery (asterisk). The white arrow indicates the general direction of migration in all panels. At E11.25 (B), the position of the wavefront has moved substantially over 16 h, and is now just rostral to the cecum. From E11.5 to E12, the position of the wavefront in the cecum changed very little over the 12-h interval although the cecum grew in diameter and the density of strands at the wavefront increased. Strands first extend from the mesenteric border (arrow) at E11.5 (C), and then the anti-mesenteric border at E12.0 (arrowhead, E). The asterisk indicates the same position in the micrographs. Scale bar = 100 μm.

extend by the caudal movement of cells within and along the strands (Young et al., 2004b; Druckenbrod and Epstein, unpublished observation). However, a strand can also extend by the addition of cells that are isolated from and caudal to the strand, described here as advanced cells (ACs). In both the ileum and caudal half of the colon, a small number of cells may transiently become ACs, but the majority of migrating cells remain interconnected and strands largely extend from within (Supplementary Movies 1 and 2). At E10.5, the wavefront in the ileum advances caudally at an average speed of

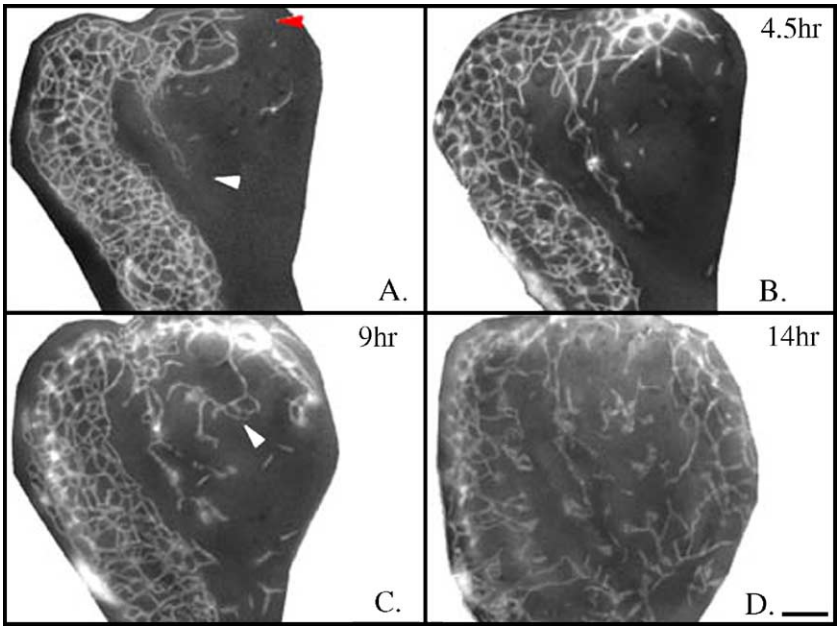

Fig. 2. The colonization of the E11.75 cecum at three different times after initiation of time lapse shows unique ENCC behavior. (A) The wavefront has bifurcated into strands seen at the apex (red arrowhead) and along the mesenteric border of the cecum (white arrowhead). (B) After 4.5 h, the density of strands at the apex has increased as cells are added from the ileo-cecal junction. The cecal body is devoid of strands although a few single cells are present. (C) After 9 h, the cecal body contains large isolated strands and a strand connected to the wavefront (arrowhead). The single cells move freely between the apex and mesenteric strands, but arise mostly from the apex. Once the apex is colonized, strands project from the anti-mesenteric border, and strands begin to project from the mesenteric border into the cecal body. (D) After 14 h, the cecal body contains a large number of isolated strands and strands connected to the network on the anti-mesenteric border of the cecum. The hitherto sparsely populated cecal body has been suddenly populated by ENCCs from the three regions. Scale bar = 100 μm.

45 μm/h (±10.8 SE, $n = 4$), but decreases to 32 μm/h at E11.25 as it approaches the nascent cecum (Figs. 1A, B). These values are similar to those reported by Allan and Newgreen (1980) in the chick.

At E11.5, the wavefront has reached the base of the developing cecum (Figs. 1C–E). For about 8–12 h, ENCCs are concentrated near the cecal base, and do not migrate caudally, but do show rapid, erratic movement. During this period, the number of strands of the paused population increases, and a small group of cells migrate as a discrete strand along the mesenteric border towards the proximal colon (Fig. 2). Eventually, the ENCCs restricted to the cecal base gradually migrate as strands towards the cecal apex (anti-mesenteric side of cecum). From this group, a number of single ACs "break-off" from the strands and enter the cecal body. After approximately 12 h, the ACs connect throughout the cecal body, and begin to extend as short strands towards one another to form a network still isolated from the larger apex population. At this time, the initial wavefront, which has now curved around the cecal apex, merges with the burgeoning network in the cecal body. The addition of strands from the cecal body results in the rapid repositioning of the inter-connected wavefront from the cecal apex to the cecal–colonic junction (Supplementary Movie 3). From E11.5 to E12.5, the wavefront advances through the caudal axis of the cecal body at an average speed of only 23 μm/h (±6.3 SE, $n = 6$). This value includes the period when the cells were paused. About 5–6 h after invasion of the cecal body, strands from the cecum begin to enter the colon.

As previously described, while the cells in the cecum have paused from E11.5 to E12.0, a long strand of cells from the cecal base invades the colon along the mesenteric border. Over time, a large number of ACs fragment from this dorsal mesenteric strand and occupy positions along the proximal colon. A second source of ACs in the proximal colon is from the dorsal mesentery (Fig. 3). Although we have not traced these cells, we suspect that they may traverse the mesentery from the ileum between E10.5 and E11.25 (Fig. 1, asterisks).

Over the next 12 h, the ACs in the proximal colon aggregate, proliferate, and expand into isolated strands. At this time, the cecal population begins to enter the colon. Eventually, the cecal population approaches and meets the colonic population; this merger results in the extension of the wavefront and the reestablishment of a complete network (Fig. 3, Supplementary Movie 4). This network is similar to that seen from E10.5 to E11.25 in the ileum, where ACs are few, and the wavefront advances by strand extension from within. From E12.5 to E13.25, the wavefront moves at an average speed of 30 μm/h (±7.8 SE, $n = 5$) along the caudal axis of the mid-colon. Fig. 4 summarizes the colonization of the cecum and proximal colon.

At E13.5, the enteric cells approach the termination of the bowel. Over the next 18 h, strands gradually extend to the terminal colon. Near the termination of the bowel, the wavefront of vagal ENCCs approaches the pelvic primordium (PP) populated by the sacral crest (Kapur, 2000). The contribution of the sacral crest to the mouse ENS has been a source of controversy. We report here the convergence of these two crest populations (Fig. 5). A small number of strands from the PP extend rostrally, just before the vagal crest arrive, and merge with the ENCCs from the vagal crest (Supplementary Movie 5).

ENCC number influences cell interaction and migratory behavior

We found that at E11.5, a number of ACs are localized along the proximal colon. While these cells exhibit a prolonged

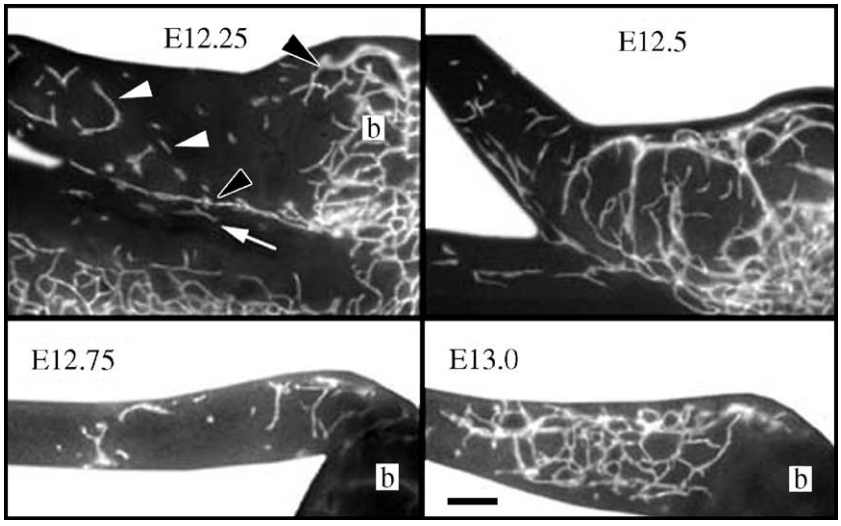

Fig. 3. Colonization of the E12.25 proximal colon. At E12.25, isolated small strands (white arrowheads) and single cells in the proximal colon appear caudal to the network of strands that colonize the cecal body (b). Distinct strands are visible on the mesenteric and anti-mesenteric borders (black arrowheads). Cells are also found in the mesentery (white arrow) E12.5. At E12.5, strands have connected the cecal body, the anti-mesenteric, and the mesenteric ENCC populations. At E12.75 (different explant), the proximal colon contains a large number of ACs arranged as short strands, single cells, and aggregates. The cecal wavefront begins to approach the ACs, as seen by the strands that have emerged from the cecal body. At E13.0, the strands from the cecum have joined the ACs, resulting in the rapid extension of the wavefront through the proximal colon. Distal strands now extend into the remaining uncolonized colon. Scale bar = 100 μm.

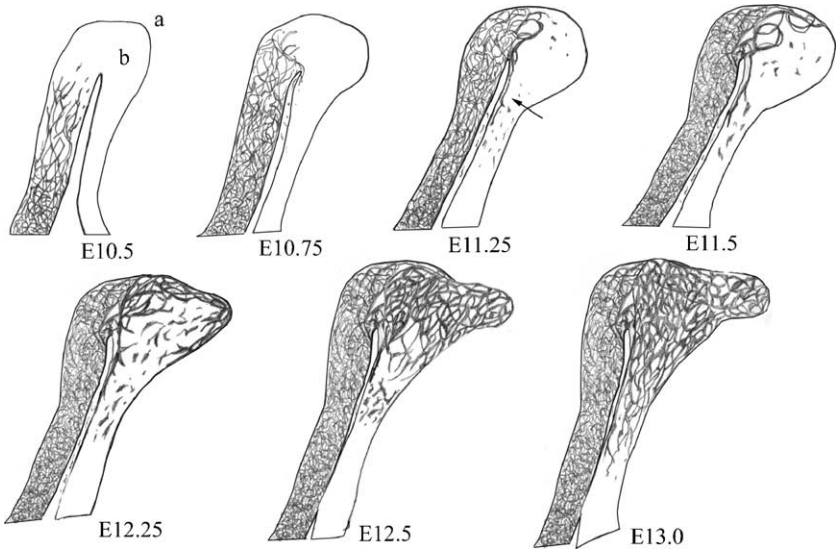

Fig. 4. Summary diagram of colonization of cecum and proximal colon with approximate embryonic ages. Distal ends of strands form a wavefront that advances through the caudal ileum at E 10.5 and reaches the ilio-cecal junction at 10.75. At this stage, a few ENCCs are found in the mesentery. From 10.75 to E11.25, most ENCCs have paused, and have accumulated at the cecal base. A few form a long strand along the mesenteric border of the cecum and colon (arrow at E11.25; mesenteric strand). Close to the termination of the mesenteric strand, isolated ENCCs appear in the caudal cecum. At E11.5, strands of cells are half way to the apex, and at E12.25, they have filled the apex and are located on the anti-mesenteric border of the cecum. At this stage, isolated clusters and solitary cells appear in the body of the cecum and are distinct from those found in the proximal colon. At E13.0, the cecal population has intersected with the clusters in the proximal colon; this merged population forms strands of ENCCs that proceed to colonize the remainder of the colon in a manner similar to that seen in the ileum.

reduction in migration, they aggregate, and then regain their migratory capacity in the form of extending strands as their numbers increase. We tested the capacity of a very small number of these ENCC to form strands and a network. In E12.5 gut, we cut the distal ends of extending strands to reduce the population distal to the cut to 2–4 cells. In such cases, the remaining strand initially retracted and then aggregated. Over the next day, the cells erratically migrate over short distances back-and-forth from one another. After 48 h, the result is typically a small aggregate or short strand of about 2–4 cells that have not migrated significantly from their initial position. This behavior suggests that aggregates of ENCCs do not form advancing strands unless a sufficient number of cells are present.

Because the pattern of migration into the cecum and proximal colon is so different from that in other gut regions, we performed experiments to determine whether reductions in cell number would alter the pattern and extent of migration. Previous observations indicated that substantial reductions in cell number result in a failure to colonize the most caudal gut regions (Burns et al., 2000; Peters-van der Sanden et al., 1993). To determine if a decrease in cells entering the cecum will alter the pattern and extent of cecum and colon colonization, we severed the cecum from the ileum and followed the colonization in the isolated gut (Figs. 6A, C1). A substantial number of ENCC had already entered the cecum and proximal colon prior to cut C1, and the cut had no effect on strand formation or the pattern of cell migration into the cecum or proximal colon (Fig. 6B). To determine if the number of advanced isolated cells in the proximal colon is sufficient to colonize the colon, we cut E11.5 gut at the cecal–colonic junction. This prevented the cecal population from joining the advanced colonic cells (Figs. 6B, C2). The colonic ACs behaved as previously described by first aggregating, proliferating, and then extending into strands. These strands formed a network whose only irregularities were an uneven distribution of cells, and regions of increased

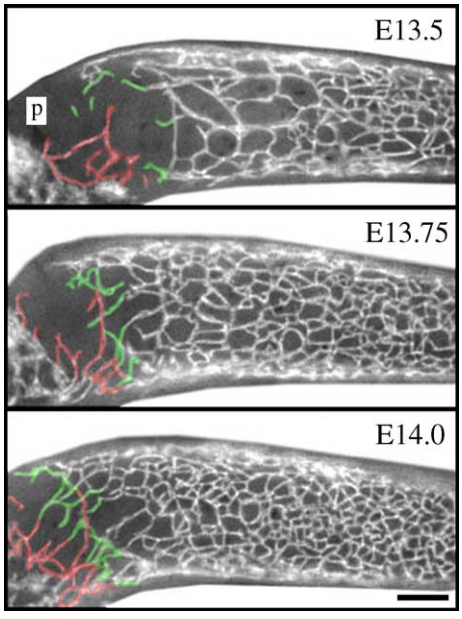

Fig. 5. Migration into the E13.5 terminal colon and the contribution of the sacral crest. At E13.5, the wavefront of vagal crest (green) is separated from the strands of sacral crest (red) emerging from the pelvic primordium (p). At E13.75, strands from both the vagal and sacral wavefront extend and intersect. By E14.0, the strand density has increased along the length of the colon and the number of intersections of between vagal and sacral strands has increased. Scale bar = 100 μm.

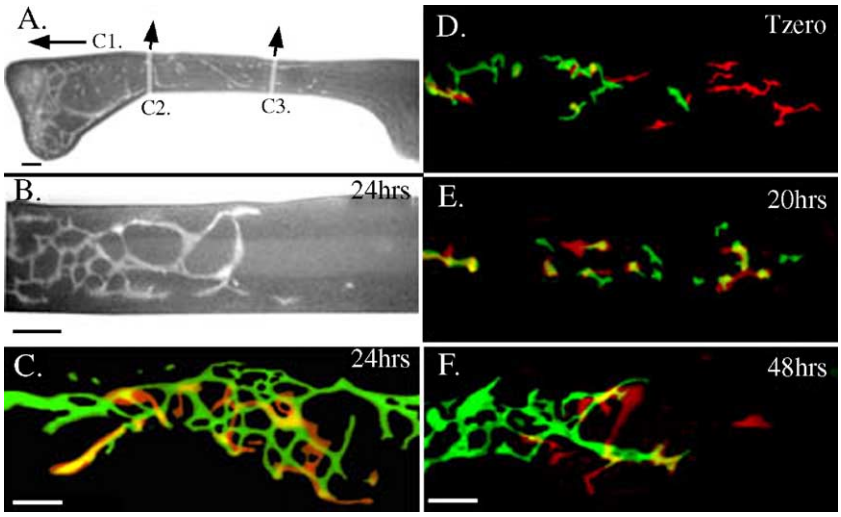

Fig. 6. Effect of cell number on migration. (A) Cuts were made in different preparations of E11.5 gut at either the ileo-cecal junction (C1), at the cecal–colonic junction (C2), and in the mid-colon (C3). (B) 24 h after the cut at C1, the ENCC wavefront advanced to the mid-colon, or approximately between C2 and C3. ENCC advance was unimpeded; the cecal wavefront advanced as described, merged with the colonic ACs, and continued to extend caudally into uncolonized colon. (C) ENCCs caudal to cut C2 developed and were isolated from the cecal wavefront. These cells developed into strands 24 h later in the mid-colon. Separation from the cecal wavefront results in a slightly diminished network rostrally (left), and strand thickness appears increased. To illustrate total ENCC distribution, both sides of the gut were imaged. The green strands are found on one side of the colon, and the red strands on the other. (D–F) Time points of ENCCs caudal to cut C3 in the hindgut. (D) Image of ENCCs caudal to cut C3 is shown at Tzero. At the start, ENCCs are well dispersed on both sides of the gut, and exist as short strands and single cells. (E) 20 h later, the cells have aggregated into a more clustered formation. (F) At 48 h, the once aggregated ENCCs have now extended into a relatively thick network predominantly on the green side of the gut. Scale bar = 50 μm.

strand thickness (Fig. 6C). This network of ENCC did advance down the colon although we did not determine whether the entire colon was colonized. Cuts in the mid-colon (C3 in Fig. 6A) resulted in fewer isolated advanced cells caudal to the cut site (Fig. 6D). These few cells aggregate and then move very little for ~20 h (Fig. 6E). Eventually, the cell groups extend into strands and begin to advance towards the terminal bowel (Fig. 6F). However, the resulting network is smaller and consists of large aggregates and very few strands. The results of these cuts (Summarized in Table 1) show that the advanced colonic population can form strands, establish a network, and initiate colonization of the colon in the absence of the cecal population.

Discussion

We have investigated the pattern of ENCC migration in the cecum, proximal, and terminal colon of the embryonic mouse gut. The cecum is of particular interest because this is the first site where defects in colonization are observed in the lethal spotted mouse model of Hirschsprung's disease (Coventry et al., 1994). In the ileum and caudal colon, strand extension appears to be a continuous process with most of the cells arising from rostral positions and advancing caudally on the strands. In the cecum and proximal colon, however, the process shows three distinct differences from the pattern observed in other regions. These differences are the pause in migration at the ileo-cecal border, the invariable route of strands into the cecum, and the extensive fragmentation of strands resulting in large numbers of advanced isolated cells (ACs).

In the ileum and caudal colon, strands of ENCCs pause for minutes but never for a period extended over hours as at the cecal base. In addition, while the trajectory of individual strands advancing through the ileum and caudal colon appears random (Young et al., 2004b), strands in the cecum invariably project along the mesenteric and anti-mesenteric faces of the

Table 1
Cell number influences the behavior of ENCCs

Age/number	Cut location	Result
E11.5 (n = 4)	(C1) Cut at ileo-cecal junction after colonization underway.	After 24 hours, there was no significant effect on the rate of cecal wavefront advance. The cellular network was unchanged from controls.
E11.5 (n = 3)	(C2) Cut at cecal-colonic junction after colonization underway.	After 24 hours, the colonic ACs developed into an unevenly distributed network, and extended strands caudally. Strand thickness was increased in areas.
E11.5 (n = 3)	(C3) Cut at mid-colon with strands caudal to cut.	Cells aggregated and remained so for about 20 hours. After 48 hours, the cell groups extended strands and advanced to the terminal bowel.
E12.5 (n = 4)	Cut at caudal end of leading strand to leave 2-4 cells caudal to cut	Remaining strand retracts and disassembles. Then cells interacted erratically by migrating short distances back and forth from each other. After 48 hours, cells had formed either a small aggregate, or a narrow, short strand.

cecum before colonizing the cecal body. Lastly, ACs in the ileum are transient and sparsely distributed close to the wavefront (Young et al., 2004b). In contrast, ACs in the cecum and proximal colon are numerous, found far from the wavefront. It is not clear what causes an ENCC to fragment from a strand to become an AC, but it appears to be a stochastic event associated with the colonization process, the incidence of which greatly increases in the cecal region. It is likely that some aspect of the unique signaling environment found in the cecum may influence or overcome the mechanisms crucial to strand integrity. Remarkably, the ACs in the proximal colon persist for hours, and while the cecum is slowly being colonized, the ACs in the proximal colon aggregate towards each other, proliferate, and then expand into short strands towards each other. As a result, once the ENCC population colonizing the cecum moves into the proximal colon, it merges with a nascent network formed by the ACs. The addition of this advanced population of ENCCs to the distal strands extending from the cecum effectively extends the wavefront caudally. The importance of adding an advanced population to the delayed cecal population is unknown, but it reestablishes an unbroken network of ENCCs that advance through the remaining colon.

At E13.5, these cells approach the sacral crest found in the pelvic primordium of the terminal colon. We have found that a small number of sacral crest cells move rostrally from the pelvic primordium into uncolonized colon and merge with the vagal crest network before complete colonization of the gut. The small number of cells is consistent with the small number of sacral crest observed by Kapur (2000) although we cannot rule out a continued contribution from the sacral crest to the network in the terminal colon. Although Kapur (2000) did not observe β galactosidase positive sacral crest independent from vagal crest in the hindgut in either Wnt1-lacZ or DβH-nlacZ embryos, this result probably reflects a limitation in not being able to observe the entire migratory process.

Our observations indicate that the distance between the sacral and vagal wavefront is very small such that the vagal ENCCs are within a few hundred microns of the termination of the bowel before the sacral crest begin to move rostrally from the pelvic primordium. Thus, there is a brief time window when the two populations are distinct. We had the ability to watch the sacral crest-derived cells emerge over time whereas Kapur would have had to fix embryos at precisely the moment when the sacral crest emerges.

The unique behavior of ENCCs in the cecum is of special interest because the expression of a number of signaling molecules is limited to or increased within cecum mesenchyme. Signaling molecules found only in the cecum include Fibroblast growth factor 10 (FGF-10), which is necessary for the normal outgrowth of the cecal epithelium (Burns et al., 2004), and Bone morphogenetic protein 4 (BMP4), which enhances the development of enteric neurons and reduces the size of the progenitor pool (Chalazonitis et al., 2004). The cecum also produces relatively high levels of message for the Glial-derived neurotrophic factor (GDNF; Young et al., 2001; Natarajan et al., 2002) and Endothelin-3 (ET-3; Leibl et al., 1999). GDNF is a chemo-attractant for enteric crest cells (Young et al., 2001), and while ET-3 itself does not attract ENCCs, it does inhibit the ability of GDNF to do so (Barlow et al., 2003; Kruger et al., 2003). ET-3 also increases the levels of GDNF expression in astrocyte cultures (Koyama et al., 2003a,b), and inhibits the differentiation of neurons in crest cultures (Wu et al., 1999). Given the unique signaling environment of the cecum, we speculate that any number of scenarios may explain the pausing behavior that we observed. For instance, the ENCCs may encounter high levels of GDNF at the cecal base and be unable to escape its attraction for 12 h, until the ET3 reduces the attraction of the ENCCs to GDNF. Alternatively, ENCCs migrating along a gradient of GDNF may become 'trapped' near the GDNF source where a meaningful gradient may be lost. Lastly, considering how the cecal wavefront shapes around the developing endoderm over time (not shown), the endoderm may be a source for some molecule restrictive of ENCC migration.

As most of these putative guidance molecules are produced by non-crest cells, the cecum illustrates the potential influences of molecules from non-crest cells on ENCC behavior. Genetic and pharmacological perturbations have revealed the complete ENCC colonization of the gut requires, and/or is influenced by certain levels of these signaling molecules (Young et al., 2001; Natarajan et al., 2002; Jiang et al., 2003; reviewed in Newgreen and Young, 2002a,b; Young et al., 2004a). Yet, the presumed influence of these signaling molecules within the unperturbed gut remains subtle. Therefore, the regular and distinctive pattern of ENCC colonization into the cecum provides an excellent opportunity to correlate ENCC behavior with endogenous changes in signaling molecule levels.

In addition to signaling molecules derived from the splanchnic mesenchyme, the migration of ENCCs may occur in part through self-regulatory mechanisms. Previous in vitro studies have shown that ENCCs population pressure reinforces migration (reviewed in Newgreen and Young, 2002b) and that individual crest cells undergo contact-stimulated migration (Thomas and Yamada, 1992). While it is difficult to resolve these presumed interactions of single cells within a strand, the influence of ENCC interaction on behavior appears significant, as deduced from the observed interactions between ACs in the cecum and proximal colon. In situ, ACs at low-density aggregate as if attracted towards each other, and then extend from each other after the aggregate reaches a sufficiently high density.

To characterize the importance of cell density on ENCC behavior, we cut the developing gut to determine whether reductions in cell number affected the pattern of colonization and migratory behavior. Young et al. (2004b) have shown that isolated strands of ENCCs show diminished migration in the gut. However, by observing the diminished cells populations over a longer time, we found that reduced populations also undergo aggregation, proliferation, and resumption of migration by strand extension. At E11.5, cuts at the ilio-cecal junction did not alter the unique pattern, or extent of migration, into the cecum and proximal colon. This result is similar to that of Sidebotham et al. (2002)

who concluded from fixed tissue sections that a small number of cells in the proximal colon at E11.5 are sufficient to colonize the colon. Furthermore, when E11.5 ACs in the proximal colon are isolated by cutting caudal to the cecum, the cells are capable of forming a caudally-advancing network. As the number of ACs decreases with cuts near the E11.5 mid-colon, the cells show very little migratory activity until their number has expanded to a critical size. When a cut reduces the number to 2–4 cells, they move erratically, but fail to expand into strands and caudal migration ceases. Thus, there is a direct relationship between migratory capacity and cell number. However, diminished cell number does not account for the unique behavior in the cecum, but reemphasizes the importance of the non-crest-derived signaling environment on ENCC colonization.

Acknowledgments

We are indebted to Dr. David Rowitch for the Wnt1-cre mice, Dr. Stuart Orkin for the Rosa-GFP mice, and Dr. Frank Costantini for the Rosa-YFP mice. We thank Dr. Paul Conner and Mr. Paul Focke for their assistance in the early phase of this work. We are grateful to Dr. Edward Schultz for reviewing the manuscript and Ms. Colleen Lavin for technical support. This study was funded by NIH NS 31385, DK 57018, and the University of Wisconsin Graduate School.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2005.08.040.

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