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Endothelial cells promote migration and proliferation of enteric neural crest cells via β1 integrin signaling

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Abstract

Enteric neural crest-derived cells (ENCCs) migrate along the intestine to form a highly organized network of ganglia that comprises the enteric nervous system (ENS). The signals driving the migration and patterning of these cells are largely unknown. Examining the spatiotemporal development of the intestinal neurovasculature in avian embryos, we find endothelial cells (ECs) present in the gut prior to the arrival of migrating ENCCs. These ECs are patterned in concentric rings that are predictive of the positioning of later arriving crest-derived cells, leading us to hypothesize that blood vessels may serve as a substrate to guide ENCC migration. Immunohistochemistry at multiple stages during ENS development reveals that ENCCs are positioned adjacent to vessels as they colonize the gut. A similar close anatomic relationship between vessels and enteric neurons was observed in zebrafish larvae. When EC development is inhibited in cultured avian intestine, ENCC migration is arrested and distal aganglionosis results, suggesting that ENCCs require the presence of vessels to colonize the gut. Neural tube and avian midgut were explanted onto a variety of substrates, including components of the extracellular matrix and various cell types, such as fibroblasts, smooth muscle cells, and endothelial cells. We find that crest-derived cells from both the neural tube and the midgut migrate avidly onto cultured endothelial cells. This EC-induced migration is inhibited by the presence of CSAT antibody, which blocks binding to β1 integrins expressed on the surface of crest-derived cells. These results demonstrate that ECs provide a substrate for the migration of ENCCs via an interaction between β1 integrins on the ENCC surface and extracellular matrix proteins expressed by the intestinal vasculature. These interactions may play an important role in guiding migration and patterning in the developing ENS.

Key words: enteric nervous system, endothelial cells, blood vessels, Hirschsprung's disease, integrins, avian, zebrafish

Introduction

The enteric nervous system (ENS) is comprised of vagal and sacral neural crest cells (NCCs) that migrate long distances along defined pathways, proliferate extensively, differentiate into neurons and glial cells, and form two concentric plexuses of interconnected ganglia in the intestinal wall. Proper migration and patterning of enteric neural crest cells (ENCCs) is essential in order to form a functional ENS capable of regulating the coordinated peristaltic activity of the gut. Interactions between ENCCs and the surrounding intestinal microenvironment are critically important. Such interactions occur between the receptors Ret and endothelin receptor B (ednrB), both expressed on the ENCC surface, and their respective ligands, glial-derived neurotrophic factor and endothelin-3, present in the intestinal mesenchyme (Heuckeroth, 2003). These interactions regulate multiple aspects of ENS development, including ENCC migration, proliferation, and differentiation. However, little is known about the signals that control migration and pattern formation in the ENS.

One important component of the intestinal microenvironment is the extracellular matrix (ECM), with which ENCCs are known to interact (Chalazonitis et al., 1997; Newgreen and Hartley, 1995; Rauch and Schafer, 2003). The ECM matrix of the intestine contains several macromolecules, including fibronectin, laminin, collagens, and tenascin, which are thought to play important roles during ENS development. For example, excessive accumulation of laminin and type IV collagen is found in endothelin-3-deficient mice and may contribute to the development of colonic aganglionosis in these mice (Gershon, 1995; Rothman et al., 1996). Among the major receptors for ECM proteins are the integrins, a large family of heterodimeric (α/β) cell surface receptors. Integrins are present on ENCCs and mediate essential cell-matrix interactions during ENS formation. Loss of $\beta 1$ integrins from the ENCC surface results in migration defects and distal colonic aganglionosis in mice (Breau et al., 2006).

ECM proteins comprise a rich network of molecules expressed throughout the gut wall. While interactions with ECM molecules are required for ENCC migration, how the ubiquitously expressed ECM matrix directs the ordered migration of these cells is unclear. Blood vessels are a potential source of signals to migrating ENCCs. During embryogenesis, endothelial cells (ECs) and nerve cells are often closely apposed (Weinstein, 2005). Both cell types undergo extensive morphogenesis to form highly organized functional networks, which often overlap in the location of their arborization (Carmeliet and Tessier-Lavigne, 2005). The major axon guidance cues - semaphorins, netrins, slits, and ephrins – also function in vessel guidance and morphogenesis (Weinstein, 2005), possibly accounting for their similar anatomic distribution (Bates et al., 2003; Carmeliet and Tessier-Lavigne, 2005; Hinck, 2004). Throughout development, ECs elaborate signals that promote neuronal survival, proliferation, migration, aggregation, and differentiation in the central (Cleaver and Melton, 2003; Leventhal et al., 1999) and peripheral (Mompeo et al., 2003) nervous systems. Based on these observations, we hypothesized that the intestinal microvasculature may similarly provide signals to ENCCs to promote and direct their migration through the intestine.

At early stages of ENS development in the avian midgut and hindgut, we find ECs forming two concentric rings that presage the patterning of the later-arriving ENCCs. As they arrive, ENCCs migrate adjacent to ECs in both avian and zebrafish embryos. Inhibition of EC development in cultured avian intestine prevents ENCC

migration, suggesting that ECs may act as a necessary substrate to guide their migration. Using organotypic cultures, we show that endothelial cells are able to promote the migration of ENCCs. This effect is abrogated by the presence of CSAT, an antibody that blocks binding to β1 integrins expressed on the ENCC surface. Together, these results demonstrate that ECs serve a critical role during ENS development by providing a substrate for the migration of ENCCs via a β1 integrin-dependent process.

Materials and Methods

Animals

Fertilized White Leghorn chicken and quail (*Coturnix coturnix japonica*) eggs were obtained from commercial breeders and maintained at 37°C in a humidified incubator. Embryos were staged according to Hamburger and Hamilton (HH) tables (Hamburger and Hamilton, 1992) or the number of embryonic days (E). Zebrafish (*Danio rerio*) embryos were raised and maintained as described (Westerfield, 1993). For immunostaining and imaging, embryos were treated with 0.003% phenylthiourea from 8 hours post-fertilization to inhibit pigment formation. The *Tg(fli1:EGFP)*^{y1} and HuC:EGFP transgenic lines were described previously (Lawson and Weinstein, 2002; Park et al., 2000). Confocal imaging of embryos was performed as described (Yaniv et al., 2006).

Enteric neural crest cell migration within the gut.

To study migration of ENCCs along the intestine, E5 (HH27) quail gut was dissected from the umbilicus to the cloaca and embedded in a three-dimensional collagen gel matrix, prepared as described (Nagy and Goldstein, 2006). The nerve of Remak was removed using tungsten needles. The collagen gel was supplemented with SU5416 (1 μM; Calbiochem; n=24). After 3 days, guts were removed and processed for immunohistochemistry.

In vitro migration of neural crest cells from neural tube and midgut.

NCC migration was assayed using explanted E5 (HH26) midgut and E2 (HH15) neural tube from chick embryos. Neural tube, extending from the level of somites 3-20, was explanted from stage HH15 chicks as described (Bronner-Fraser, 1996; Newgreen and Murphy, 2000). Briefly, embryos were treated with dispase II (2 mg/ml in DMEM; Roche) for 10 min. at 37°C. After several washes, the neural tube was dissected using tungsten needles and explanted onto plastic tissue culture dishes coated with fibronectin (20 μg/ml; Sigma), laminin (10 μg/ml; Sigma), HepG2 human hepatoma cells (ATCC: HB8065), or human umbilical vein endothelial cells (Huvec).

To assess ENCC migration from the intestine, E5 (HH26) midgut was isolated and plated onto plastic dishes, primary chick smooth muscle cells, primary chick embryonic fibroblasts, Huvec, laminin, or fibronectin. Primary Huvec cultures were obtained from human umbilical vein and used within three passages. They were confirmed to be endothelial based on characteristic morphology and CD31 (Santa Cruz Biotech) immunoreactivity. Primary chick fibroblasts and smooth muscle cells were prepared from chick embryonic limb buds and gizzards, respectively, as described (Nagy et al., 2001). Neural tube and midgut cultures were maintained for 20 hours in DMEM

containing 10% FCS, glutamine, non-essential amino acids, 100 U/ml penicillin-G, and 0.1 mg/ml streptomycin mixture. Cultures were supplemented with the monoclonal antibodies CSAT (20 μg/ml), a β1 integrin blocking antibody (Testaz et al., 1999), or BoA1 (20 μg/ml), an anti-avian B lymphocyte antibody (Igyarto et al., 2008), as indicated.

Immunohistochemistry

Avian guts were fixed in 4% formaldehyde, gelatin-embedded, frozen, and sectioned at 7-10 μm onto poly-L-lysine slides (Sigma). Primary antibodies used are listed in Table 1. Sections were incubated with primary antibodies for 45 min., followed by biotinylated goat anti-mouse IgM or IgG (Vector Labs, Burlingame, CA) and avidin-biotinylated peroxidase complex (Vectastain Elite ABC kit, Vector Labs). Endogenous peroxidase activity was quenched by incubation for 10 min. with 3% hydrogen peroxide (Sigma). The binding sites of the primary antibodies were visualized by 4-chloro-1-naphthol (Sigma) or diaminobenzidine (DAB). Erythrocytes were visualized using DAB-based peroxidase histochemistry.

For double-immunofluorescence, sections were incubated with primary antibodies for 45 min. Fluorescent secondary antibodies included: Alexa Fluor 594 anti-mouse IgG, Alexa Fluor 488 anti-rabbit, Alexa Fluor 594 anti-mouse IgM, Alexa Fluor 488 anti-mouse IgM, anti-mouse IgG isotype 1-Alexa-488 (Molecular Probes), and anti-mouse IgG Pacific Blue (Jackson ImmunoResearch). Cell nuclei were stained by DAPI (Vector Labs). For semithin (1 μm) sections, tissue was fixed in 4% paraformaldehyde and embedded in Polybed/Araldite 6500 (Polysciences Inc). HNK-1 staining was visualized with 3,3'- diaminobenzidine-NiSO4 (Sigma) and QH1 staining was visualized with Novared (Vector Labs). Images were compiled using Adobe Photoshop.

For wholemount immunofluorescent staining, E5 and E6 quail intestines were fixed with 4% formaldehyde for 3 hours. After several washes in PBS, the specimens were incubated overnight with primary antibodies (QH1 and HNK1) followed by secondary antibodies (Alexa Fluor 594 anti-mouse IgG and Alexa Fluor 594 anti-mouse IgM) for 5 hours.

Zebrafish embryos were fixed overnight in 4% paraformaldehyde at 4°C. For immunofluorescence, embryos were hydrated through graded methanol series, washed in PBT (PBS + 0.01% Tween), and blocked in 5% goat serum in PBT. Staining of enteric neurons was performed using a 1:25 dilution of anti-HuC/D in blocking solution. GFP on endothelial cells was detected using a 1:400 dilution of anti-GFP antibody (Molecular Probes). Alexa Fluor 546 goat anti-mouse and Alexa Fluor 488 goat anti-rabbit (Molecular Probes) were used as secondary antibodies.

Immunoelectron microscopy

Sections from E8 (HH36) quail hindguts were fixed in 4% paraformaldehyde in PBS for 48 hours, then infiltrated with 7.5% gelatin in PBS for 2 hours at 37°C. The gelatin cube was postfixed in 4% paraformaldehyde and 50 μm sections incubated with HNK-1 antibody for 24 hours, followed by goat anti-mouse IgM (Vector Laboratories) for 24 hours and avidin- biotinylated peroxidase complex for 6 hours (Vectastain Elite ABC kit, Vector Laboratories). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide (Sigma). HNK-1 staining was visualized with 3,3'- diaminobenzidine-

NiSO4 (Sigma). Extensive washing in PBS for 3 hours was followed by avidin-biotin blocking (Vector Laboratories) and addition of the second primary antibody (QH1) for 24 hours, biotinylated goat anti- mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL) for 24 hours, and Streptavidin-Gold (10nm, Sigma). After immunohistochemistry, samples were postfixed in 4% glutaraldehyde for 24 hours and 1% osmium tetroxide (Polysciences Inc., Warrington, PA) for 2 hours. After dehydration in graded ethanol, tissue blocks were embedded in Polybed/Araldite 6500 (Polysciences Inc.) and ultrathin (60 nm) sections were contrasted with uranyl acetate and lead citrate and studied with a Hitachi electron microscope type H-7600.

Results

ENCCs follow endothelial cells as they migrate along the gut.

Blood vessel development in the avian intestine was examined using QH1, a marker of quail ECs (Pardanaud et al., 1987), and HNK-1, a marker for ENCCs. ECs are seen in the submucosal and myenteric layers of the distal E5 (HH27) midgut at a time when only the myenteric plexus has been colonized by ENCCs (Fig. 1A,B). The ECs and ENCCs are immediately adjacent to each other in the myenteric region. At E6 (HH30), the submucosal plexus of the distal midgut is starting to form and again the close relationship between ECs and ENCCs is evident (Fig. 1C,D). In the E5 (HH27) and E6 (HH30) hindgut, which are still preganglionic, ECs are already present in two concentric rings within the intestine (Fig. 1E,F). As ENCCs colonize the hindgut at E7 (HH33; Fig. 1G) and E8 (HH36; Fig. 1H), QH1 and HNK-1 double-staining demonstrate that the position of the ECs predicts the patterning of the arriving ENCCs, which reach the mid-colorectum at least 2 days after the appearance of ECs. By E8 (HH36), ENCCs and ECs are adjacent to each other in two organized concentric rings (Fig. 1H). The relationship of the migrating ENCCs to the ECs is seen in a longitudinal section of E7 (HH33) hindgut which captures the migratory front of ENCCs in the submucosal plexus (I, arrow) as they travel proximo-distally (left-to-right) along the ECs. Higher magnification confirms that ECs and ENCCs are in direct contact with one another, as shown on a semithin (1 μm) section of an E8 (HH36) hindgut (Fig. 1J). The presence of erythrocytes (Fig. 1J, arrows) within the QH1-immunoreactive cells confirms that this structure represents a blood vessel. The anatomic proximity of the two cell types is also evident in the interplexus fibers extending between submucosal and myenteric ganglia at later stages (Fig. 1K).

Immunoelectron microscopy of E8 (HH36) quail hindgut demonstrates cell-cell contact between EC and ENCC (Fig. 1L-N). After double immunohistochemistry on vibratome sections from E8 (HH36) quail hindgut, ultrathin sections were made for electron microscopy. HNK-1 staining was detected with DAB-NiSO4, which stains as a black precipitate, and QH1 was detected with strepavidin-conjugated 10 nm gold particles. As outlined in Fig. 1L, and magnified in Fig. 1M,N, an HNK-1+ ENCC is found in contact with a QH-1+ EC that lines a small blood vessel in the intestinal wall. To quantitatively validate the association between ENCCs and ECs, the total number of HNK-1+ ENCCs and the number in contact with QH-1+ ECs was counted under standard light microscopy in each of 5 sections at different stages during colorectal colonization. At all stages examined, from E6.5 (HH31-32), when the migratory wavefront is at the

proximal colon, until E8 (HH36), when colorectal colonization is complete, at least 80% of ENCCs appear to be in contact with ECs.

Wholemount immunohistochemistry was performed using QH1 and HNK-1 antibodies. QH1+ endothelial cells form a network within the gut wall, with ENCCs and neurites interposed (Suppl. Fig. 1A). ECs and ENCCs are adjacent to each other in this single-plane image. This is better seen at higher power (Suppl. Fig. 1B), confirming the cross sectional data. In Suppl. Fig. 1C, processes are seen extending from an ENCC near the migratory wavefront onto nearby endothelial cells, suggesting a possible role for ECs in guiding migration.

Endothelial cells are required for ENCC migration.

To determine whether blood vessels are necessary for ENCC migration, EC development was inhibited using SU5416, a pan-VEGFR inhibitor (Tille et al., 2003) that blocks the receptor's tyrosine kinase activity (Mendel et al., 2000). Control E5 (HH27) quail gut explants were cultured in collagen gel with no additive for 72 hours (Fig. 2A). At E5 (HH27), Hu+ ENCCs are present only in the distal midgut (Fig. 2B), while QH1-immunoreactive ECs are present along the length of the intestine (Fig. 2C). After 3 days in culture, ENCCs migrate to the distal colorectum (Fig. 2D,E) and QH1-immunostaining shows a normal distribution of ECs (Fig. 2F). Cross sections through the hindgut reveal ENCCs and ECs present in two concentric rings at this stage (Fig. 2G,H). Culturing the gut in the presence of SU5416 leads to distal intestinal aganglionosis, with enteric crest cell migration arrested at the distal midgut (Fig. 2I,J). This migratory arrest was observed consistently in all guts examined (n=20). SU5416 treatment causes endothelial cells to form sparse and disorganized clusters of cells (Fig. 2K-M), lacking the density and patterning seen in normal intestine (Fig. 2F,H). Importantly, at the concentration used, SU5416-treated guts appeared histologically normal, other than for the endothelial cell changes, including normal smooth muscle actin staining (not shown). Aganglionosis in these treated guts is confirmed by the absence of HNK-1-immunoreacitvity (Fig. 2N). The level of aganglionosis varies with the stage at which the inhibitor is added. Addition of SU5416 to E6 (HH30) intestine stops migration at the proximal hindgut (not shown).

Neural crest cell migration onto cultured ECs is dependent on β1 integrin.

Based on the above experiments, we hypothesized that ECs serve as a substrate for the migration of ENCCs. We first tested this hypothesis on pre-migratory ENCCs. Chick neural tube from somite levels 3-20, which includes the vagal and truncal regions, was isolated and cultured onto untreated plastic for 20 hours. Minimal cell migration was observed in these cultures (Fig. 3A). Culturing onto a laminin- or fibronectin-coated surface, however, led to robust migration of HNK-1-immunoreactive NCCs (Fig. 3B), as previously reported (Bronner-Fraser, 1986; Newgreen and Thiery, 1980). After 20 hours in culture, more ENCCs appear to migrate onto the fibronectin surface, as compared to laminin, as has been observed previously (Desban and Duband, 1997). To test the ability of NCCs to migrate onto a cellular surface, HepG2 cells, an epithelial cell line derived from the liver, were grown to confluence and the neural tube cultured onto the dish. No crest cell migration was observed (Fig. 3C). However, when neural tube was cultured onto a layer of Huvec cells, brisk migration of vagal-level HNK-1+ cells occurred onto the endothelial cell surface (Fig. 3D). This migration was inhibited by the addition of

CSAT, a β1 integrin function blocking monoclonal antibody (Fig. 3F), to the culture media, but not by addition of BoA1, an avian-specific anti-B lymphocyte monoclonal antibody used as a control (Fig. 3E). Since the CSAT antibody is specific for avian β1 integrin (Testaz et al., 1999), the Huvec cells are not affected. Caspase-3 immunostaining was performed to test whether the significant decrease in migrating HNK-1+ cells in the CSAT-treated cultures was due to cell death, but no evidence of apoptotic neural crest-derived cells was identified (Fig. 3F).

ECs promote migration and proliferation of ENCCs via β1-integrin binding.

In order to determine whether ECs promote migration of ENCCs within the gut, the above experiment was repeated using the intestine. E5 (HH26) chick midgut was explanted onto untreated plastic (Fig. 4A). Only a small number of HNK-1+ cells are seen migrating a short distance away from the gut. When cultured onto a confluent layer of Huvec cells for 20 hours, many HNK-1+ ENCCs migrate out of the intestine (Fig 4B). As controls, primary cultures of chick embryonic gizzard smooth muscle cells (Fig. 4C) and limb bud fibroblasts (Fig. 4D) were plated. Minimal ENCC migration was observed on these substrates. In contrast, laminin (Fig. 4E) and fibronectin (not shown) promoted brisk ENCC migration. In order to determine whether ENCC migration was occurring secondarily, in response to the migration of other intestinal cells onto the surrounding matrix, cultures were stained with QH1 and SMA (Fig. 4F). No endothelial cell migration was observed (Fig. 4F, top panel). SMA+ cells migrated out of the gut a short distance, but the HNK-1+ ENCCs migrated further (Fig. 4F, bottom panel), suggesting that they were not migrating in response to the SMA+ cells. The addition of CSAT monoclonal antibody inhibited ENCC migration onto laminin (Fig. 4G) and Huvec cells (Fig. 4H), while normal migration occurred in the presence of control BoA1 monoclonal antibody (not shown). Caspase-3 immunoreactivity again revealed no increase in ENCC apoptosis following CSAT treatment compared to control (not shown).

CSAT can inhibit ENCC migration either by reducing the number of cells migrating out of the gut or by reducing the distance that those cells migrate. In order to look at this, the number of HNK-1+ ENCCs migrating out of the intestine in Huvec cultures was counted in 5 high-powered fields. In control Huvec cultures, 213 ± 30 ENCCs migrated, while in the presence of CSAT, only 23 ± 18 ENCCs migrated ($p<0.05$). The distance migrated was also significantly affected, with ENCCs migrating 4652 ± 629 μm in control Huvec cultures and 1087 ±243 μm in the presence of CSAT. Inhibition of β1 integrin by CSAT thus inhibits both the number of ENCCs migrating and the distance migrated.

To determine whether the presence of ECs in the culture influences ENCC proliferation, E5 (HH26) midgut was cultured either on laminin or on Huvec cells for 20 hours, followed by double immunofluorescence with HNK-1 and phosphohistone-H3 (Ser10) to detect mitotically active ENCCs (Fig. 4I, arrows). To quantitate the difference, the percentage of HNK-1+ ENCCs that were phosphohistone-H3+ was determined in several different cultures. The percentage of dividing ENCCs was 9.9 ± 6.0 and 5.7 ± 2.4 in Huvec and laminin cultures, respectively, representing a 1.7-fold increase in ENCC proliferation in Huvec cultures ($p<0.05$). A similar comparison was done between Huvec (11.6 ± 3.2) and fibronectin (4.0 ± 1.9), yielding a 2.9-fold increase in ENCC

proliferation in Huvec cultures ($p<0.001$). These results demonstrate that ECs promote both migration and proliferation of ENCCs.

Laminin is strongly expressed around blood vessels.

The inhibitory effect of CSAT antibody on ENCC migration and proliferation suggests an essential role for matrix proteins in mediating these EC-induced effects. The expression of ECM proteins was examined in sections of E8 (HH36) hindgut. As shown in Fig. 5B-E, tenascin, type III collagen, fibronectin, and laminin are all strongly and ubiquitously expressed in the intestinal mesenchyme. Expression is absent in the epithelium, neuronal plexuses, and nerve of Remak. While laminin is expressed throughout the mesenchyme (Fig. 5E), the strongest immunoreactivity is observed surrounding blood vessels, which are identified by the presence of peroxidase-positive erythrocytes within the vessel lumen (Fig. 5F). Immunofluorescent staining for laminin preferentially marks this strong laminin expression on blood vessels, and shows this vessel-associated laminin to be closely associated with HNK-1+ ENCCs (Fig. 5G). CSAT antigen, known to be expressed on the surface of NCCs (Krotoski et al., 1986), is strongly expressed on ENCCs (Fig. 5H,I), confirmed by its colocalization with HNK-1 (Fig. 5J,K).

ENCCs migrate along blood vessels in the zebrafish gut.

To determine if a similar relationship between ENCCs and ECs is observed in other vertebrates, zebrafish embryos were examined using a transgenic line in which all endothelial cells are labeled with GFP. *TG(fli1:EGFP)* zebrafish (Lawson and Weinstein, 2002) were allowed to develop for 2-6 days post-fertilization (dpf). As in avian embryos, the intestinal vessels of the zebrafish are present prior to the appearance of enteric neurons (Suppl. Fig. 2). Subintestinal vessels can be seen by 2 dpf (Suppl. Fig. 2B), well before the appearance of Hu+ cells in the mid-intestine at 3 dpf (Suppl. Fig. 2E). The relative position of ECs and ENCCs was examined at 6 dpf, after completion of ENS colonization of the gut (Shepherd et al., 2004). As seen in Fig. 6B-D, Hu-immunoreactive enteric neurons are located along the GFP-labeled subintestinal vessels in wild-type embryos. The close proximity between endothelial and enteric neuronal cells is observed both in the mid-intestine (Fig 6C) and posterior intestine (Fig. 6D). In order to test whether ENCCs are preferentially located near ECs in zebrafish embryos, we developed a software tool to compare statistically the observed arrangement of ENCCs to the "null hypothesis" of random assortment of ENCCs relative to the adjacent blood vessels (Suppl. Fig. 3). This analysis revealed that the juxtaposition observed between ENCCs and blood vessels is non-random and highly significant, with a p-value=0.00001. While a close relationship between vessels and enteric neurons is evident at 6 dpf (Fig. 6), further studies will be needed to evaluate this relationship at the migratory wavefront in the zebrafish gut.

Discussion

ENCCs migrate along defined pathways to form the highly patterned ENS, which consists of two concentric rings of ganglia on either side of the circular smooth muscle layer of the intestine. The signals driving migration and patterning in the ENS are largely

unknown. No factor has been identified that displays the selective expression pattern required for a guiding role in ENCC migration. We have found an important spatiotemporal relationship between the development of the intestinal microvasculature and the migratory path of ENCCs. Well before the arrival of migrating ENCCs, ECs are distributed in two concentric rings within the gut mesenchyme, presaging the patterning of enteric ganglia. As ENCCs arrive, they are closely associated with the blood vessels, as observed in both avian and zebrafish embryos. This relationship suggests that signals arising from the blood vessels may regulate migration of these neural crest-derived cells. Following inhibition of blood vessel formation in the gut, ENCC migration is arrested, leaving the distal intestine aganglionic, confirming the essential role of the microvasculature in ENS formation.

The endothelial basement membrane is a strong candidate to serve as a substrate for ENCC migration. NCCs are known to be in contact with basement membranes lining their migratory pathways along the neural tube, somite, and myotome (Duband and Thiery, 1987; Perris et al., 1991; Tosney et al., 1994), even demonstrating a preference for migration along basal laminae (Tosney et al., 1994). Basement membranes are rich in ECM components, which guide the directional migration of NCCs during multiple aspects of organogenesis (Perris and Perissinotto, 2000). We tested the ability of endothelial cells to promote the migration of premigratory ENCCs by explanting the neural tube, including the vagal region, onto a variety of surfaces, including untreated plastic, laminin, fibronectin, endothelial cells, and epithelial cells. As expected, NCCs migrated avidly onto laminin and fibronectin, both of which are known to serve as substrates for migration of neural crest-derived cells (Desban and Duband, 1997; Lallier and Bronner-Fraser, 1991; Newgreen, 1984; Newgreen and Thiery, 1980). While no migration occurred onto the epithelial cell surface, Huvec cells served as an excellent substrate, promoting brisk migration of NCCs away from the neural tube. This result is consistent with previous work implicating blood vessels in guiding the early migration of NCCs away from the neural tube (Erickson, 1985; Newgreen and Thiery, 1980; Spence and Poole, 1994). We next tested whether endothelial cells can stimulate a similar response from migratory ENCCs in the intestine by culturing E5 (HH26) chick midgut onto various substrates, including ECM proteins, fibroblasts, smooth muscle cells, and endothelial cells. Fibronectin and laminin again produced robust ENCC migration out of the intestine, as did Huvec cells. Importantly, when compared to fibronectin and laminin, Huvec cultures promoted significantly more ENCC proliferation than either ECM component, indicating that endothelial cells are capable of supporting both the migration and proliferation of ENCCs.

The effects of ECs in ENS development may be mediated by various mechanisms, via factors expressed on their surface or via secreted molecules that signal to nearby ENCCs. Artemin, for example, is a neurotrophic factor expressed on blood vessels that guides sympathetic neurons to their targets (Honma et al., 2002). Alternatively, ECs secrete matrix metalloproteinases to promote neuroblast migration (Wang et al., 2006), or brain-derived neurotrophic factor to drive neuronal migration and survival (Leventhal et al., 1999; Louissaint et al., 2002). ECM proteins secreted by endothelial cells may also be responsible for mediating the effects on ENCCs. Given the known importance of the ECM during NCC (Perris and Perissinotto, 2000) and ENS development (Newgreen and Hartley, 1995), we tested whether ECM interactions were

required for endothelial cell-mediated migration. We tested this using CSAT, a monoclonal antibody that binds the β1 subunit of the integrin receptor (Bronner-Fraser, 1986; Neff et al., 1982) and blocks binding by laminin, fibronectin, and type IV collagen (Hall et al., 1987; Horwitz et al., 1985), all of which are expressed in the endothelial cell basement membrane (Fridman et al., 1985; Payette et al., 1988). CSAT antigen is strongly expressed on ENCCs (Fig. 5K), making this a good candidate for inhibition. We have shown that addition of CSAT antibody to cultures of midgut plated on Huvec cells significantly inhibited ENCC migration onto the surrounding cellular substrate. These results support our conclusion that β1 integrins present on the ENCC surface interact with ECM molecules produced by ECs to promote cell migration and proliferation in the developing ENS.

During ENS development, ENCCs migrate in an environment rich in fibronectin, laminin, type IV collagen, and tenascin (Newgreen and Hartley, 1995; Payette et al., 1988; Rauch and Schafer, 2003). Laminin in the extracellular milieu of the gut binds to a laminin binding protein on the surface of ENCCs (Chalazonitis et al., 1997; Payette et al., 1988; Pomeranz et al., 1991). The importance of this ENCC-ECM interaction is supported by data from mice with colorectal aganglionosis due to endothelin-3 mutations. The aganglionic region in these mice accumulates excessive amounts of laminin (Payette et al., 1988; Rothman et al., 1996), which may promote premature neuronal differentiation and thereby lead to the cessation of ENCC migration (Gershon et al., 1993). Interestingly, laminin binding protein is a non-integrin binding molecule and therefore should not be affected by CSAT, suggesting that both integrin and non-integrin mediated interactions with the ECM may be required for ENS development. Abnormal expression of laminin and type IV collagen has previously been described in human Hirschsprung's disease (Parikh et al., 1992), further emphasizing the role of the ECM in ENS development. Recent evidence supporting the critical importance of ENCC-ECM interactions comes from targeted deletion of β1 integrins on crest-derived cells. Mice harboring this mutation exhibit distal colorectal aganglionosis (Breau et al., 2006). Why the aganglionosis is limited to the distal gut is unclear. However, since ENCCs express other integrins, such as α4 (Kruger et al., 2002), and also non-integrin ECM receptors, such as laminin binding protein (Chalazonitis et al., 1997), these may partially compensate for the loss of β1. The results presented suggest that interactions between β1 integrins on the surface of migrating ENCCs and endothelial-derived ECM molecules are critically important for migration of crest-derived cells during ENS development. Future investigations will be directed toward identifying the molecular interactions that occur and the mechanisms by which ECs exert their effects.

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Figure Legends

Figure 1. Endothelial cells presage the patterning of ENCCs in the embryonic gut. Quail midgut (A-D) and hindgut (E-K) were stained with antibodies to QH1 (red), HNK-1 (green in A-C,E-I,K or blue in D,J), and SMA (blue in B). Longitudinal (A, distal end down, lumen to left) and transverse (B) sections of E5 (HH27) midgut show myenteric ENCCs adjacent to ECs. ENCCs arrive in the submucosal plexus at E6 (HH30;C), adjacent to the endothelial plexus already present. Semithin sections of E6 (HH30) midgut show the close relationship between ENCC (blue) and EC (red) in both submucosal (D, top) and myenteric (D, bottom) plexuses. In the hindgut, ECs are present in two concentric rings as early as E5 (HH27;E) and E6 (HH30;F) in a pattern predictive of the later arriving crest-derived cells, which reach the mid-colorectum at E7 (HH33;G) and form two complete plexuses adjacent to ECs by E8 (HH36;H). Longitudinal section of E7 (HH33) hindgut (I; distal end to right, luminal side down), shows the migratory wavefront (arrow) of ENCCs colonizing the myenteric plexus. Semithin section of E8 (HH36) hindgut shows EC (red) and ENCC (blue) in contact (J), with erythrocytes (J, arrows) seen within the vessel. The proximity of ECs and ENCCs is evident at E12 (HH40), even in the inter-plexus region (K, boxed area magnified in inset). Immunoelectron microscopy of E8 (HH36) hindgut demonstrates contact between HNK-1+ ENCCs (outlined in blue) and QH1+ ECs (outlined in yellow). A leukocyte (L) is present within the capillary lumen. The boxed areas in L are magnified in M and N, where QH1+ endothelial cells are marked with gold particles (M, arrows). ENCC, enteric neural crest cell; EC, endothelial cell; L, leukocyte; M, mesothelial cell; mp, myenteric plexus; smp, submucosal plexus. Scale bar in A corresponds to 130 μm (A), 100 μm (B,C), 15 μm (D), 100 μm (E), 130 μm (F), 120 μm (G), 150 μm (H), 45 μm (I), 7 μm (J), 100 μm (K), 70 μm (K, inset), 10 μm (L), 2 μm (M,N).

Figure 2. Endothelial cells are required for ENS development. As depicted schematically in (A), E5 (HH27) quail gut was explanted and cultured in collagen gel for 3 days. Longitudinal sections of wild-type E5 (HH27) intestine stained with Hu show ENCCs present only in the midgut (B), while QH1-labeled ECs are present throughout the intestine (C). After 3 days in culture, ENCCs have migrated to the distal colorectum, as seen on longitudinal sections stained with Hu (D) and HNK-1 (E), with normal QH1 staining present (F). Transverse sections through the mid-hindgut labeled with HNK-1 (G) and QH1 (H) demonstrate ENCCs and ECs, respectively, in two plexuses within the gut wall. The addition of SU5416 to the culture leads to arrested ENCC migration at the level of the ceca (I,J), with markedly abnormal EC development (K, boxed area magnified in L). Sections through the hindgut at the level marked in (I) show sparse and abnormally arranged QH1-stained cells (M) and complete absence of ENCCs (N). hg, hindgut; mg, midgut; wt, wild-type. Scale bar in panel N corresponds to 0.5 mm (B-C), 0.7 mm (D), 0.4 mm (E,F), 140 μm (G,H), 0.5 mm (I-K), 130 μm (L), 100 μm (M,N).

Figure 3. Migration of NCCs on endothelial cells is inhibited by CSAT. Stage HH 15 chick neural tube was explanted onto untreated (A), laminin-coated (B, top panel), or fibronectin-coated (B, bottom panel) petri dishes for 20 hours. While little NCC migration is observed on the untreated surface (A), brisk migration occurs onto laminin

and fibronectin. No NCC migration was observed on cultures of HepG2 cells (C), while Huvec cultures stimulated NCC migration out of the gut (D). Huvec-induced migration was inhibited by addition of CSAT (F), but not by addition of a control monoclonal antibody, BoA1 (E). The inhibition by CSAT is not associated with significant cell apoptosis, as shown by the paucity of caspase-3+ cells (F, arrowheads). nt, neural tube.

Figure 4. EC-induced migration of ENCCs requires β1 integrin binding. E5 (HH26) chick midgut was placed onto the surface of an untreated plastic dish (A) or onto confluent Huvec cells (B), primary chick smooth muscle cells (C), primary chick fibroblasts (D), or laminin-coated plastic (E). Migration of ENCCs onto the surrounding environment after 20 hours was detected with HNK-1 antibody (red). Migration of intestinal ECs and smooth muscle cells onto laminin was detected using QH1 and SMA antibody, respectively (F). CSAT inhibits ENCC migration onto laminin (G) and Huvec (H). Proliferating ENCCs were identified by colocalization of HNK-1 and phosphohistone-H3 (Ser10) immunoreactivity (I, arrows and inset). mg, midgut. Scale bar in A corresponds to all panels.

Figure 5. Laminin-expressing endothelial cells are adjacent to CSAT-expressing ENCCs. Serial sections through an E8 (HH36) chick hindgut were stained with HNK-1 (A), tenascin (B), type III collagen (C), fibronectin (D), and laminin (E). Laminin is strongly expressed on vascular endothelial cells (F, dark blue), identified by the presence of peroxidase-positive erythrocytes (F, brown). HNK-1+ ENCCs (G, red) are closely associated with these laminin+ vessels (G, green). CSAT immunostaining labels cells in the submucosal and myenteric plexuses of the E8 (HH36) hindgut (H,I). Double immunofluorescence labeling confirms the colocalization of HNK-1 (J and K, red) with CSAT (J and K, green). ep, epithelium; NoR, nerve of Remak; mp, myenteric plexus; smp, submucosal plexus.
Scale bar in A corresponds to 200 μm (A-E,H), 30 μm (F,G,I,K), 100 μm (J).

Figure 6. Enteric neural crest cells align with blood vessels in the zebrafish gut. (A) Diagram of a zebrafish embryo with red box depicting the approximate area of the gut imaged in panels B-D. (B) Hu+ ENCCs (red) are seen in close proximity to EGF-labeled ECs (green) in 6 dpf *TG(fli1:EGFP)* wild-type embryos (white arrows). This colocalization is present in both mid- (C) and posterior intestine (D).

Supplemental Figure 1. Endothelial cells and ENCCs form a rich network within the intestinal wall. Double wholemount immunohistochemistry was performed on E6 quail midgut using QH1 (red) and HNK-1 (green) antibodies. A network of ECs and ENCCs is present in the intestine (A), with ECs and ENCCs in close proximity to one another (B). Near the migratory wavefront, ENCC processes are seen extending onto nearby ECs (C).

Supplemental Figure 2. Subintestinal vessels (SIVs) form ahead of enteric neurons in zebrafish embryos. (A) Diagram of a zebrafish embryo with red box depicting the approximate area of the yolk extension, gut, and trunk imaged in panels B-I. Confocal images of corresponding areas of *fli:EGFP* (B,D,F,H) and HuC:EGFP (C,E,G,I)

transgenic embryos at the same stages of development. At 2 dpf, the subintestinal vascular network is seen along the yolk extension (B, arrow), whereas no enteric neurons are detected in this area at the same developmental stage (C). At 3 dpf, a few enteric neurons (E, arrow) have reached the dorsal aspect of the yolk extension. At 4 dpf (F,G) and 6 dpf (H,I), larger numbers of neurons have arrived, and the SIVs and ENS cells now surround a well-formed intestine. In panels E,G,I, confocal stacks were used to specifically identify ENCC-derived GFP signal in the background of green yolk autofluorescence. Hu-labelled cells were then manually colored red in reconstructed images to facilitate visualization. dpf, days post-fertilization; y.e., yolk extension; SIVs, subintestinal vessels. The image in panel A is modified from (Kimmel et al., 1995).

Supplemental Figure 3. The juxtaposition of ENCCs and vessels in the zebrafish intestine is non-random. Gut region of a *Tg(fli:EGFP)* embryo at 5dpf, showing enteric neurons (A,C; HuC, red) and subintestinal vessels (B,D; EGFP, green). Neurons (C) and vessels (D) of each imaged gut (n=10) were traced using a software tool developed in Matlab © for the purpose of quantifying the proximity of enteric neurons to endothelial cells. Briefly, the distance of each neuron from its proximal blood vessel is computed. The tendency of the neurons to concentrate in the vicinity of the blood vessels is measured by the sum of these distances (Sum of Shortest Distances, or SSD). We tested the significance of the non-uniform spatial distribution of the neurons by estimating the probability distribution function of the SSD for uniformly distributed neurons (shown as bell-shaped curve in E) and comparing it to the experimentally observed SSD, noted by the arrow on the graph in panel (E). The random distribution was computed by keeping the number of neurons fixed (equal to the experimentally measured number), repeatedly assigning random coordinates to the neurons to simulate uniform spatial distribution, and then computing the SSD for each distribution, performing a total of 100,000 repetitions. The software reports a summary of the process (right of panel E), with information on the number of neurons and blood vessels, number of random repetitions, observed SSD, and p-value for the comparison between the random distribution and our experimental results.

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Cells/structures identified	Antibody specificity	Species specificity	Antibody	Source
Neural crest cells	3-sulphoglucuronosyl antigen	chick / quail	clone HNK-1 (mouse IgM)	NeoMarkers
Neural crest cells	β1 integrin	chick / quail	Clone CSAT (mouse IgG2b)	DSHB
Neurons	HuC/D	chick / quail	Clone 16A11 (mouse IgG2b)	Molecular Probes
Extracellular matrix	laminin	chick / quail	Clone 31 (mouse IgG1)	DSHB
Extracellular matrix	fibronectin	chick / quail	Clone B3/D6 (mouse IgG2a)	DSHB
Extracellular matrix	tenascin	chick / quail	Clone MI-B4 (mouse IgG1)	DSHB
Extracellular matrix	collagen type III	chick	Clone 3B2 (mouse IgG1)	DSHB
Hematopoietic and endothelial cells	alpha-macroglobulin	quail	Clone QH1 (mouse IgG1)	DSHB
Apoptotic cells	caspase-3	chick / quail	Rabbit polyclonal	NeoMarkers
Proliferating cells	phosphohistone-H3 (Ser10)	chick / quail	Rabbit polyclonal	Millipore
Smooth Muscle Cells	Alpha-smooth muscle actin (SMA)	chick / quail	Clone 1A4 (mouse IgG2a)	Dako
B lymphocytes	Bu-1 antigen	chick / quail	Clone BoA1 (mouse IgG1)	Igyarto et al., 2008

Table 1. Primary Antibodies.

DSHB, Developmental Studies Hybridoma Bank