Analysis of the Sacral Neural Crest Cell Contribution to the Hindgut Enteric Nervous System in the Mouse Embryo

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BACKGROUND & AIMS: The majority of the enteric nervous system is derived from the vagal neural crest, with a second contribution, which is restricted to the post-umbilical gut, originating from the sacral neural crest. In mammals, although sacral neural crest cells (NCCs) have been shown to enter the hindgut, information on their development and role remains scant. Our aim was to determine the migratory routes of sacral NCCs to the hindgut, their timing and site of entry into the gut, and their migratory behaviors and differentiation within the hindgut. **METHODS:** We used in situ cell labeling, whole embryo culture, immunofluorescence, organotypic culture, and time-lapse live-cell imaging in mouse embryos. **RESULTS:** Sacral NCCs emigrated from the neural tube at embryonic day 9.5, accumulated bilateral to the hindgut to form prospective pelvic ganglia at embryonic day 11.5, and from there entered the distal hindgut through its ventrolateral side at embryonic day 13.5. They then migrated along nerve fibers extending from the pelvic ganglia toward the proximal hindgut, intermingling with rostrocaudally migrating vagal NCCs to differentiate into neurons and glia. In organotypic culture, genetically labeled sacral and vagal NCCs displayed different capabilities of entering the hindgut, implying differences in their intrinsic migratory properties. Time-lapse live-cell imaging on explants ex vivo showed that sacral NCCs migrated along nerve fibers and exhibited different migratory behaviors from vagal NCCs. **CONCLUSIONS: Murine sacral NCCs are a distinct group of cells that migrate along defined pathways from neural tube to hindgut. They exhibit discrete migratory behaviors within the gut mesenchyme and contribute neurons and glial cells to the hindgut enteric nervous system.**

Keywords: Cell Migration; Pelvic Ganglia; Gut Explant Culture; Live Cell Imaging.

The enteric nervous system (ENS) is responsible for regulating many important gut functions, including peristalsis.[1] Studies on chick and rodent embryos have shown that the intrinsic neurons and glia that comprise enteric ganglia within the gut are derived from the neural crest.[2] The majority of neural crest cells (NCCs) that colonize the gut originate from the vagal level of the neuraxis adjacent to somites 1 to 7.[2–5] Another smaller but significant contribution that is restricted to the post-umbilical ENS comes from a second, caudal region of the neuraxis, the sacral neural crest.[3–8] Although the contribution of sacral NCCs to the post-umbilical ENS, and particularly the terminal hindgut, has been shown conclusively in chick embryos, information regarding sacral NCCs in mammals, including humans, is still scant and fragmentary. Importantly, the hindgut, defined as the second half of the transverse colon, descending colon, and rectum, is the most common site of congenital ENS abnormalities, such as Hirschsprung disease[9] and intractable constipation,[10] which lead to dysfunction of the intestine and can be lethal or life-threatening. Thus, there is a need to better understand the development and role of sacral NCC within the mammalian hindgut. Studies using 1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate as an exogenous marker indicated that in the mouse, sacral NCCs contributing to enteric ganglia emigrate from the neural tube at the neuraxial level caudal to somite 24.[11] With the use of another cell marker, wheat germ agglutinin-gold conjugates (WGA-Au), our previous studies showed that NCCs caudal to somite 24 started their migration from the neural tube at around embryonic day (E) 9.5 and these early migrating cells mainly traversed dorsoventrally through the somatic mesenchyme between the somite and the neural tube.[12] Results of experiments using different genetic markers, kidney capsule grafting, and time-lapse videos[13–16] also indicated that mouse sacral NCCs may follow similar migratory pathways to those in the chick. All these studies suggest that mouse sacral NCCs caudal to somite 24 start to emigrate from the neural tube at E9.5 and take about 4 days before they begin to appear within the hindgut mesenchyme at E13.5 to E14.0. However, their precise migratory routes and the exact timing and site of entry to the hindgut remain to be elucidated. Here, we mapped the entire migratory pathway of mouse sacral NCCs from the neural tube to the hindgut, determined the timing and the axial levels they enter the hindgut, and examined their migratory behaviors and fate within the hindgut.

Abbreviations used in this paper: E, embryonic day; ENS, enteric nervous system; GFP, green fluorescent protein; NCC, neural crest cell; ss, somite stage; WGA-Au, wheat germ agglutinin-gold conjugate.

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Material and Methods

Animals

Green fluorescent protein (GFP) mice (C57BL/6-Tg[ACTB-EGFP]10sb/J; Jackson Laboratory, Bar Harbor, ME), in which the ubiquitous expression of GFP was driven by the β-actin promoter,[17] and normal Institute of Cancer Research mice (Harlan, Oxfordshire, UK) were used. The morning when a vaginal plug was identified was designated as E0.5. All experimental protocols and animal handling procedures adopted in the present study were approved by the Animal Experimentation Ethics Committee (ref. no. 08/020/ERG and 461808) of the Chinese University of Hong Kong and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Sacral NCC Labeling, Whole Embryo Culture, and Reconstruction of Images

Pregnant mice were sacrificed at E9.5 and embryos dissected. A dark-red solution of WGA-Au[18,19] was microinjected into the lumen of the sacral neural tube caudal to somite 24 (Figure 1*A*). The labeled embryos were cultured in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA) with 20% heat-inactivated rat serum and 1% penicillin/streptomycin, as described previously,[12,20] then processed for silver enhancement staining[18,21] after serial transverse sectioning. Reconstruction of images was performed with Neurolucida software (Microbrightfield Inc, San Diego, CA).

Tissue Recombination in Organotypic Explant Culture

Explants of gut segments and isolated pelvic ganglia were recombined ex vivo and cocultured in an organotypic culture system.[22] Briefly, the hindgut, with a short pre-cecal midgut segment (about 0.5 cm in length), was isolated from Institute of Cancer Research mouse embryos at E11.5 in Dulbecco's modified Eagle medium/F12 medium (Invitrogen) to serve as the recipient tissue. Pelvic ganglia and segments of the rostral half of the hindgut plus a short pre-cecal midgut segment were isolated from GFP mouse embryos at E12.5 and were used as the donors of sacral and vagal NCCs, respectively. One recipient hindgut segment was then transferred to an organ culture dish (Falcon; Becton Dickinson, Franklin Lakes, NJ) containing culture medium (Dulbecco's modified Eagle medium/F12 medium containing 0.12% sodium bicarbonate, 10% fetal bovine serum, and 1% penicillin/streptomycin). The 2 ends of the gut segment were fixed to an agarose block (4%) by fine tungsten needles (Fine Science Tools, Foster City, CA). A GFP-labeled donor pelvic ganglion or a segment of the rostral half of the donor hindgut was apposed to the ventrolateral or dorsal side of the recipient hindgut. Recombined explants were cultured with 5% CO_2 at 37°C for 2–3 days.

Time-Lapse Confocal Microscopic Live-Cell Imaging

One to 2 days after culture, the recipient gut together with the attached donor tissue was transferred to a glass culture dish filled with culture medium, which was then put in a culture chamber (Tokai Hit Co, Shizuoka-ken, Japan) at 37°C supplied with 5% CO_2 on the stage of a confocal microscope (Olympus FV-ASW1.6) equipped with time-lapse, multifluorescence recording software (FV10-ASW ver.01.07). The cultured explants were scanned with a 20× objective every 20 minutes. Each image was stacked from 10–15 optical sections of 15–20 μm taken at each time point.

Immunohistochemistry

For double or multiple immunofluorescence staining of wholemount preparations and sections antibodies listed in Supplementary Table 1 and standard procedures developed previously in our laboratory were used.[23]

Results

Early Migration Pathways of Sacral NCCs

Premigratory sacral NCCs were labeled by microinjection of WGA-Au solution into the lumen of the sacral neural tube (caudal to the level of somite 24) of mouse embryos at the 22-somite stage (ss) (E9.5) that were grown ex vivo by whole embryo culture (Figure 1*A*). The migratory pathway was reconstructed from images of serial transverse sections through the sacral region (Figure 1*B*). At the 26ss (8 h in culture, n = 5, E9.5), a few labeled cells started to emigrate from the dorsal neural tube and accumulated at the dorsal tip of the somite, mainly at the level of somites 24 and 25 (Figure 1*C*, Supplementary Figure 1*A*). At the 30ss (16 h in culture, n = 5), labeled cells were scattered among sclerotomal cells between the dermomyotome and the neural tube (Supplementary Figure 1*B*), spreading along about half of the dorsoventral extent of the neural tube at the axial levels of somites 25 to 28 (Supplementary Figure 1*B*). By the 34ss to 36ss (24 h in culture, E10.5, n = 5), labeled cells were spread dorsoventrally from the dorsal part of the neural tube to the regions around the dorsal aorta (Figure 1*D*), and many of them were scattered throughout the anterior and posterior regions of somites 24 to 30 (Figure 1*D*). No labeled cells were found emigrating from the neural tube caudal to the level of somite 33.

Neural crest–derived WGA-Au–labeled cells were characterized using p75 and Sox10 immunoreactivity, the only neural crest–specific antibodies that successfully labeled these cells from all those tested in Supplementary Table 1. Premigratory sacral NCCs residing in the neural tube were $p75^-/Sox10^+$ at E9.5 (Supplementary Figure 2), but 1 day after labeling, WGA-Au–labeled sacral NCCs from the neural tube started to express weak p75 together with strong Sox10 immunoreactivities (not shown). Some of these doubly immunoreactive cells coalesced to form dorsal root ganglia and expressed strong p75 and Sox10 immunoreactivities (Figure 1*Ei–iii*, Supplementary Figure 3*A′*). Sacral NCCs scattered among sclerotomal cells and near the dorsal aorta all showed strong double positivity (Figure 1*Ei–iii, Fi–iii*, Supplementary Figure 3*A–A′*).

Formation of Pelvic Ganglia and Entry to the Distal Hindgut

At E11.5, sacral NCCs ($p75^+/Sox10^+$) were scattered along the dorsoventral pathway extending from the neural tube through the somatic mesenchyme lateral to the dorsal aorta to the regions dorsolateral to the hindgut, where they aggregated to form the prospective pelvic