

Multipotential progenitors of the mammalian enteric nervous system capable of colonising aganglionic bowel in organ culture

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SUMMARY

The enteric nervous system of vertebrates is derived from neural crest cells that invade the gut wall and generate a highly organised network of enteric ganglia. Among the genes that play an important role in ENS development is *c-Ret*, mutations of which result in failure of formation of enteric ganglia (intestinal aganglionosis). To further understand the development of the mammalian ENS in general and the mechanism of action of the RET RTK in particular, we have developed and used an organotypic culture system of mouse fetal gut. At the stage of culture initiation, the gut is partially populated by undifferentiated ENS progenitors, but culture for several days results in extensive neuronal and glial differentiation. Using this organ culture system, we have compared the development of the ENS in wild-type and RET-deficient gut and showed that the aganglionic phenotype observed *in vivo* is

consistently reproduced under the *in vitro* culture conditions. Microinjection of RET⁺ cells isolated from E11.5 mouse bowel into wild-type or RET-deficient aganglionic gut in organ culture, results in extensive repopulation of their wall by exogenously derived neurons and glia. Finally, using a similar approach, we demonstrate that single RET⁺ cells introduced into the wall of wild-type gut generate both cell lineages of the ENS, i.e. neurons and glia. Our data show the NC-derived RET⁺ population of fetal gut in mammalian embryos consists of multipotential progenitors capable of colonising efficiently both wild-type and RET-deficient aganglionic bowel in organ culture.

Key words: Enteric nervous system, Nervous system, Neural crest, *c-Ret*, Mouse, Gut, Aganglionic bowel

INTRODUCTION

The enteric nervous system (ENS) of vertebrates is composed of a large number of neuronal and glial cells organised into two ganglionic plexuses (submucosal and myenteric) that are arranged as concentric rings in the wall of the bowel (Gershon et al., 1994). A salient feature of the vertebrate ENS, which is unique to the peripheral nervous system (PNS), is that its neurons form highly complex local reflex circuits, which function largely independently of the central nervous system (CNS) to control contractility of the smooth muscle layers of the bowel and secretions of the enteric glands (Furness and Costa, 1987). The functional complexity of the ENS is reflected in the number and phenotypic diversity of its neurons, which produce a large variety of neuropeptides and neurotransmitters (Costa et al., 1996; Gershon et al., 1994). The combinatorial expression of these molecules generates a chemical code that has been used to classify the neurons of the mammalian ENS into functionally distinct groups (Sang et al., 1997; Sang and Young, 1996). In addition to neurons, the ENS contains a large number of supporting glial cells which, unlike their counterparts in the PNS, express molecular markers, such as glial fibrillary acidic protein (GFAP), characteristic of CNS astrocytes (Jessen and Mirsky, 1980).

The ENS of vertebrates is derived from the neural crest (NC) (Le Douarin and Teillet, 1973; Yntema and Hammond, 1954). In mouse embryos, neuroectodermal precursors destined to colonise the bowel emigrate from the neural tube at the level of somites 1-7, between embryonic day (E) 8.5 and 9.0 (Durbec et al., 1996). Upon completion of their pre-enteric migratory phase and prior to entry into the foregut, the ENS precursors accumulate in a region of the embryo that lies ventral to the aorta and immediately posterior to the branchial arches (Durbec et al., 1996). From this location and under the influence of unknown signals, they enter the foregut (E9.0-9.5) and, migrating in a rostrocaudal direction, colonise the entire bowel (E9.5-12.5) (Durbec et al., 1996; Jacobs-Cohen et al., 1987; Kapur et al., 1992; Rothman and Gershon, 1982; Rothman et al., 1984). During migration, the ENS progenitors increase in number and, upon arriving at their final destination sites, they differentiate into the various types of neurons and glia of the mature ENS (Gershon and Tennyson, 1991). Several studies have demonstrated that signalling molecules derived from the mesenchyme of the gut wall play an important role in the differentiation of the ENS and that its normal morphogenesis and functional organisation are dependent on the maintenance of the three-dimensional organisation of the fetal bowel (Baetge et al., 1990; Gershon et al., 1993; Mackey et al., 1988). Thus,

formation of the ENS in mammalian embryos depends on a series of overlapping developmental processes that are controlled by a complex set of interactions between the enteric NC and the surrounding mesenchymal cells.

Genetic and molecular studies have identified several genes that play a critical role in the development of the mammalian ENS (Gershon, 1997). Among them the *c-Ret* and *Gdnf* loci encode interacting components of a single signal transduction pathway. *c-Ret* encodes the receptor tyrosine kinase (RTK) RET, which is widely expressed in the PNS, including the ENS, throughout embryogenesis (Durbec et al., 1996; Pachnis et al., 1993; Tsuzuki et al., 1995). *Gdnf*, on the contrary, encodes one of the functional ligands of the RET RTK, glial cell lined-derived neurotrophic factor (GDNF) and is expressed in the mesenchymal cells of the gut wall (Hellmich et al., 1996; Suvanto et al., 1996, and C. V. M.-G. and V. P., unpublished observations). Humans heterozygous for germline mutations at the *c-RET* locus often suffer from congenital megacolon (Hirschsprung's disease), characterised by absence of enteric ganglia from varying lengths of the colon (Ederly et al., 1994; Romeo et al., 1994), while mice homozygous for a functional deletion of *c-Ret* (*Ret.k⁻*), die at birth due to kidney agenesis and lack of enteric ganglia posterior to the cardiac stomach (intestinal aganglionosis) (Schuchardt et al., 1994). Analysis of homozygous *Ret.k⁻* embryos has demonstrated that the aganglionic gut phenotype is due to an early defect of enteric NC (Durbec et al., 1996). Consistent with the functional interaction between GDNF and RET in vivo, deletion of the *Gdnf* locus in mice results in a phenotype very similar to that of RET-deficient animals, including severe intestinal aganglionosis (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996).

Despite several genetic and expression studies, our understanding of the mechanism of action of *c-Ret* in the developing ENS is incomplete. For example, it is currently unknown whether the aganglionic phenotype of RET-deficient embryos is due exclusively to a cell-autonomous requirement of *c-Ret* function in enteric NC or whether mutations in this locus also affect, directly or indirectly, the ability of gut mesenchyme to support normal ENS development. Also, the potential association between expression and activation of the RET RTK in enteric NC cells and their commitment and differentiation to specific cellular phenotypes in vivo is largely unknown. Finally, very little is known regarding the potential role of *c-Ret* on the migration, survival or proliferation of the ENS precursors and their progeny. Given the complexity of interactions between the ectodermally derived enteric NC and the mesenchymal wall of fetal bowel, any definitive answers to the above questions require experimentation in easily accessible systems that are capable of maintaining the integrity of the bowel and thus normal interactions between its various cell types.

Here, we report on the development of an organotypic culture system of mouse fetal gut that is capable of maintaining the three-dimensional organisation of the bowel and supporting differentiation and morphogenesis of the intrinsic ENS. Using such a culture system, we demonstrate that a phenotypically defined population of wild-type enteric NC cells (expressing *c-Ret*; RET+) is capable of colonising RET-deficient aganglionic bowel and generating mature neurons and glia. Based on these findings, we suggest that *c-Ret* functions cell-autonomously in

enteric NC and that the mesenchyme of mutant gut provides all the signals that are necessary to support differentiation and morphogenesis of the ENS. Also, by introducing single cells into gut in organ culture, we show that the vast majority of RET+ cells isolated from the gut of E11.5 wild-type mouse embryos are composed of multipotential precursors capable of generating both cell lineages of the mammalian ENS (i.e. neurons and glia). These data suggest that commitment of ENS progenitors to differentiated phenotypes is taking place subsequently to their migration into the bowel and that one of the functions of the RET RTK is to promote the expansion of the originally sparse population of multipotential enteric NC entering the foregut. While our manuscript was under review, a similar organ culture system of mammalian fetal gut was reported by Young and colleagues (Young et al., 1998). The data presented here support and extend further the findings of this report.

MATERIALS AND METHODS

Animals

Mice carrying the *Ret.k⁻* mutation have been described previously (Durbec et al., 1996; Schuchardt et al., 1994). Parkes mice are wild type at the *c-Ret* locus and were maintained as an outbred colony. The generation of the PTY mouse line has been described previously (Skarnes et al., 1995).

Organ culture of mouse fetal gut

For organ culture, the gut (including esophagus, stomach and small and large intestine) was isolated from E11.5 Parkes embryos (wild type; outbred) or embryos derived from *Ret.k⁻* intercrosses in L15 medium (Life Technologies, UK) and cleaned from contaminating tissues. In our initial experiments, the dissected gut was placed in a three-dimensional collagen gel matrix as previously described (Tessier-Lavigne et al., 1988) and maintained (in 4-well plates; NUNC) in a defined medium (optiMEM; Life Technologies, UK) supplemented with L-glutamine (1 mM; Life Technologies, UK) and antibiotic mixture (100 U/ml; Life Technologies, UK) in an atmosphere of 5% CO₂. In subsequent experiments, we observed that the viability and growth of fetal gut was similar when cultured as a free-floating organ in the same medium. The results obtained with both types of cultures were identical in every respect and all experiments reported here were conducted with free-floating cultures. Individual guts were cultured in 300 µl of medium in 4-well plates (NUNC) and were fed every 2 days.

For the bromo-deoxyuridine (BrdU) incorporation experiments, 10 µM of BrdU (Boehringer-Mannheim) was added to the culture medium for 24 hours at 37°C, followed by a period of 48 hours in BrdU-free medium before fixation (Chalazonitis et al., 1994).

Isolation of RET + cells from fetal mouse gut

RET+ cells were isolated from fetal gut essentially as described by Lo and Anderson (Lo and Anderson, 1995). Briefly, guts (including esophagus, stomach, and small and large intestine) were dissected from E11.0-11.5 PTY mouse embryos in L15 medium on ice. After two washes in Ca²⁺/Mg²⁺-free PBS they were dissociated using a mixture of collagenase and dispase (0.1 mg/ml; SIGMA) for 5 minutes at room temperature. Upon further washing in Ca²⁺/Mg²⁺-free PBS, single cell suspension was generated by trituration in L15 medium (supplemented with 10% fetal calf serum, dextrose, fresh vitamin mix, glutamine and antibiotics). The cells were washed again in the same medium, counted and then incubated with a cocktail of three different hamster anti-RET hybridoma supernatants (3A61D7, 3A61C6 and 2C42H1; kindly provided by D. Anderson) for 45-60

minutes on ice. The cells were then washed in supplemented L15 medium and incubated with donkey anti-rat phycoerythrin-conjugated antibody (1:50; Jacksons ImmunoResearch Laboratories) for 30 minutes on ice in the dark. Following this incubation, the cells were washed 3× in ice-cold supplemented L15 medium and then passed through a nylon mesh to generate a single cell suspension and remove clumps. RET⁺ cells were isolated on a FACStar (Becton-Dickinson) Fluorescence Activated Cell Sorter using Argon ion laser at 488nm. Finally the cells were collected in sterile test tubes and counted. Fig. 4A,B shows a typical sorting profile. RET⁺ cells constitute 4-5% of the total population of gut cells. To confirm that the isolated cell population was enriched for *c-Ret*-expressing cells, a combination of reverse transcription and semiquantitative polymerase chain reaction (RT-PCR) was performed using primers specific for *c-Ret* mRNA sequences. Based on the intensity of the signal obtained (Fig. 4C), we estimated that the immunoreactive population of cells was enriched at least 20-fold compared to the non-reactive cells. The sequences of the primers used for RT-PCR were:

P1, 5'-TTTGAATTCGGCACAGCGCATCACACAGTGG-3';
P2, 5'-TTTGATATCGGCCTCTATTTCTCAAGGGATGC-3'.

Injection of RET⁺ cells into fetal gut in culture

RET⁺ cells (resuspended at a concentration of 50-100 cells/ 0.5 µl), were injected into the stomach wall of fetal gut using pulled-out capillary micropipettes (Clark Electromedical Instruments). For single cell injections, the RET⁺ cells were resuspended at a concentration of 0.5-1 cell/ 0.5 µl. Individual droplets (0.5 µl) were placed on Petri dishes on ice and examined under the microscope. Those containing one cell were drawn into capillary micropipettes and injected into the stomach wall of the gut. Injected explants were cultured for 7 or 14 days under the conditions described above.

Immunostaining

Immunofluorescence assays were performed on cryosections. At the end of the culture period, guts were fixed in 4% paraformaldehyde for 2 hours at 4°C and, after cryoprotection (in 30% sucrose) and embedding in OCT compound, they were sectioned at 12 µm. Incubations with all primary antibodies were carried out at 4°C overnight, while incubations with secondary antibodies were carried out at room temperature for 45 minutes. The primary and secondary antibodies are listed in Table 1. Analysis of double label immunofluorescence was performed with a Leica TCS NT Confocal microscope.

In situ hybridisation

In situ hybridisation on gut sections was performed using a *c-Ret*-specific riboprobe derived from pmcret7 as previously described (Pachnis et al., 1993).

β-galactosidase histochemistry

For X-gal staining, guts were fixed in 1% formaldehyde, 0.1% glutaraldehyde, 1 mM MgCl₂, 1 mM EGTA and 0.02% NP40 (in PBS) at the end of the culture period for 20 minutes at 4°C. β-galactosidase-expressing cells were visualised by incubating tissue samples at 37°C (overnight) in staining solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl₂, 0.01% deoxycholate and 0.02% NP40 and 1 mg/ml X-gal in PBS. The whole-mount preparations were photographed and then postfixed in 4% paraformaldehyde (in PBS) for 2 hours at room temperature, incubated in 30% sucrose (in PBS) overnight at 4°C, embedded in OCT compound and sectioned (20 µm) in a cryostat (Jung).

For detecting BrdU on X-gal-stained tissue, prior to addition of anti-BrdU antibody, sections were treated with 4N HCl for 10 minutes at room temperature, rinsed several times in Tris-Borate buffer (pH 8.5) followed by 2 washes in 1× PBS. Signal was detected using diaminobenzidine as substrate.

RESULTS

Development of the intrinsic ENS in organ cultures of mouse fetal gut

To gain further insight into the cellular and molecular mechanisms controlling the development of the mammalian ENS, we wished to establish an organ culture system of mouse fetal gut that would allow easy access and experimental manipulation of its intrinsic nervous system. For such a system to be informative upon experimental manipulation, it would require to fulfill two main criteria: first, to preserve the three-dimensional organization of the bowel and thus maintain normal cellular interactions over a relatively long culture period and, second, to support the differentiation of ENS precursors into the types of neurons and glial cells present in the mammalian gut. To establish such a system, the bowel of E11.0-11.5 wild-type mouse embryos (outbred; Parkes) was

Table 1

Antigen	Host	Dilution	Detection
Neuron-specific Enolase (NSE) (Polysciences Inc.; USA)	Rabbit	1:1000	FITC-conjugated Donkey anti-rabbit F(ab) ₂ fragments (Jacksons ImmunoResearch Laboratories) or Rhodamine-conjugated sheep anti-rabbit (Boehringer Mannheim)
Vasoactive Intestinal Peptide (VIP) (Biogenesis; UK)	Rabbit	1:100	FITC-conjugated donkey anti-rabbit F(ab) ₂ fragments
Neuropeptide-Y (NPY) (Biogenesis; UK)	Rabbit	1:400	as above
Calcitonin Gene Related Peptide (CGRP) (Biogenesis; UK)	Rabbit	1:100	as above
S-100 (Dakopatts; Denmark)	Rabbit	1:400	as above
Glial Fibrillary Acidic Protein (GFAP) (Dakopatts; Denmark)	Rabbit	1:100	as above
Anti-α-smooth muscle Actin (SIGMA Immunochemicals)	Mouse	1:400	FITC-conjugated donkey anti-mouse F(ab) ₂ fragments
Anti beta-galactosidase (SIGMA Immunochemicals)	Mouse	1:1000	Rhodamine-conjugated sheep anti-mouse (Boehringer Mannheim)
anti-BrdU (Boehringer-Mannheim)	Mouse	1:100	Horse radish peroxidase-conjugated goat anti-mouse (Dakopatts; Denmark)

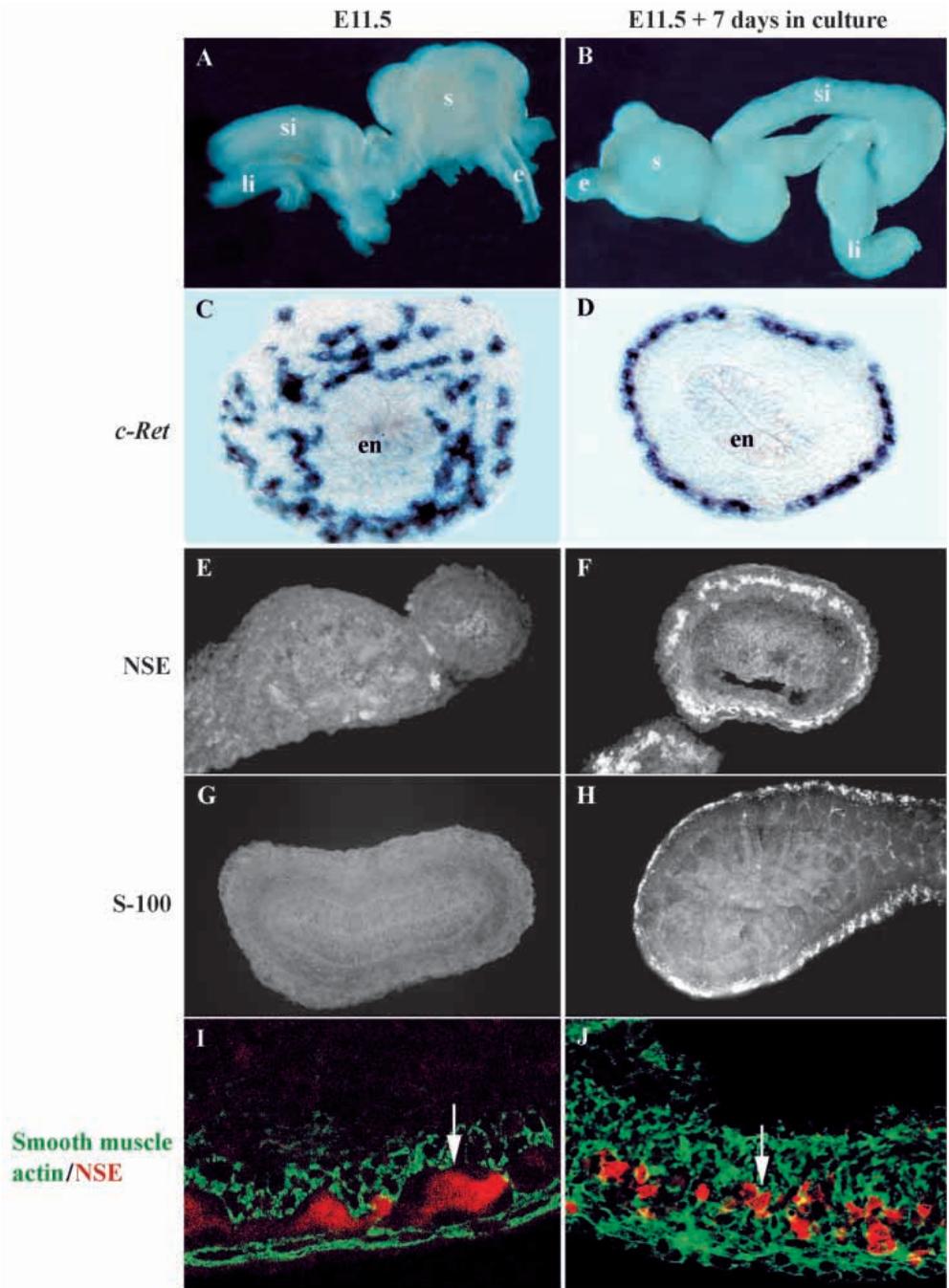


Fig. 1. Differentiation of enteric NC cells into mature neurons and glia in mouse fetal gut organ cultures. The top panels show whole guts either immediately after dissection from E11.5 mouse embryos (A) or after culturing as free-floating organs for 7 days (B). The middle panels show staining of sections from E11.5 mouse gut immediately after dissection (C,E,G) or at the end of a 7-day culture period (D,F,H) with: a *c-Ret*-specific riboprobe (C,D), NSE (E,F) and S-100 (G,H). The sections in the bottom panels were derived from E17.5 mouse embryo gut (I) or E11.5 mouse embryo gut at the end of a 7-day culture period (J) and were doubly stained with antibodies specific for smooth muscle actin and NSE. The arrows in these sections indicate the differentiated neurons embedded in the outer smooth muscle layer of the gut wall. e, esophagus; en, gut endoderm; li, large intestine; s, stomach; si, small intestine.

removed and embedded in a three-dimensional collagen gel matrix or allowed to develop as a free-floating organ in a defined tissue culture medium (for experimental details see Materials and Methods). Using either of these methods, the fetal bowel survived, increased in length and maintained its overall organization for at least 14 days, the longest period that it was allowed to develop *in vitro* (Fig. 1A,B). In addition, peristaltic activity of the gut wall commenced usually within 48 hours and was maintained throughout the culture period. No significant difference was observed between the two methods of culture (collagen gel-embedded or free-floating organ) and all the experiments reported here were performed with gut growing as a free-floating organ.

At the stage of dissection (E11.0-11.5), the gut wall was populated by NC-derived cells, as indicated by the expression of the tyrosine kinase receptor RET, one of the earliest markers of enteric NC in vertebrate embryos (Kapur et al., 1992; Lo and Anderson, 1995; Marcos-Gutierrez et al., 1997; Pachnis et al., 1993; Robertson and Mason, 1995; Schuchardt et al., 1995). Such RET⁺ cells were present in the foregut and midgut of the bowel but had only partially colonized the hindgut (Fig. 1C and (Durbec et al., 1996; Young et al., 1998)). We also observed that during the migratory phase of ENS development (E9.0-13.0), RET⁺ cells were dispersed throughout the mesenchyme of the gut wall, but at subsequent stages they organised themselves into concentric rings of

tightly compacted cells (Fig. 1C,D; see also introduction). Despite the presence of a relatively large number of RET+ cells, staining of multiple serial sections from E11.5 mouse embryo gut with antibodies specific for postmitotic neurons (neuron-specific enolase-NSE and neuron-specific class III β -tubulin-Tuj1) and mature glia (S-100), demonstrated that both differentiated cell types were absent at this stage (Fig. 1E,G and data not shown). To determine whether fetal gut in organ culture could support differentiation of the intrinsic ENS precursors, similar staining was performed on E11.5 guts at the end of a 7-day culture period. As shown in Fig. 1F,H, large numbers of neurons and glia were present throughout the length of the gut (including the entire hindgut). Interestingly, the vast majority of differentiated cells were organised into tight cellular collections at the periphery of the gut wall in a ring-like arrangement similar to the one observed in vivo (compare panels F and H to panel D of Fig. 1). Furthermore, double-label immunofluorescence experiments with antibodies specific for smooth muscle actin and NSE indicated that the position of the in vivo and in vitro differentiating neurons relative to the outer smooth muscle layer was comparable (Fig. 1I,J). Overall, our findings show that the gut of E11.5 mouse embryos is partially colonized by undifferentiated ENS precursors which, under the conditions employed here, are capable of fully colonising the gut and differentiating into mature neurons and glia, which assume a topographic organisation similar to that observed in vivo.

In addition to the pan-neuronal (NSE) and pan-glial (S-100) markers, we also examined the differentiated progeny of enteric NC for expression of genes that define specific subpopulations of enteric neurons or mature ENS glia. For this, the gut of E11.5 mouse embryos was dissected and allowed to develop as a free-floating organ for 14 days in defined medium. At the end of the culture period, cryosections of gut explants were stained with antibodies specific for various neuropeptides (such as neuropeptide Y-NPY, vasoactive intestinal peptide-VIP and calcitonin gene-related peptide-CGRP), as an index of neuronal diversity and for GFAP, as a characteristic marker of mature enteric glia (Gershon et al., 1994; Jessen and Mirsky, 1980). As shown in Fig. 2, culture of fetal gut for 2 weeks leads to the appearance of neurons expressing all the neuropeptides tested and GFAP-positive glia. These data suggest that, in addition to the differentiation of neurons and glia at the appropriate location in the gut wall, organ cultures of fetal gut cultures can also support development of the complex pattern of gene expression that is normally associated with mature mammalian ENS.

Organ culture of RET-deficient mouse fetal gut

Mice homozygous for a mutation at the *c-Ret* locus (*Ret.k⁻*) have severe intestinal aganglionosis (Durbec et al., 1996; Schuchardt et al., 1994). To determine whether this phenotype can be reproduced in vitro, organ cultures of gut were established from E11.5 mouse embryos derived from intercrosses of *+Ret.k⁻* heterozygous animals. As expected, a large number of neurons and glia developed in the gut from wild-type (*+/+*) or heterozygous (*+Ret.k⁻*) embryos (Fig. 3A,B and data not shown). In contrast, gut from homozygous mutant (*Ret.k⁻/Ret.k⁻*) embryos failed to generate both neurons and glia (Fig. 3C,D) [except for very few cells that were generally restricted to the esophagus and cardiac stomach (data not shown)]. This in vitro phenotype is consistent with the severe intestinal aganglionosis observed in vivo (Durbec et al., 1996; Schuchardt et al., 1994) and indicates that the organ culture conditions used here were unable to rescue the aganglionic phenotype of RET-deficient gut.

RET+ cells from mouse fetal gut can colonise wild-type and aganglionic gut in vitro

Grafting experiments in avian embryos have shown that NC derivatives from embryonic bowel were capable of migrating

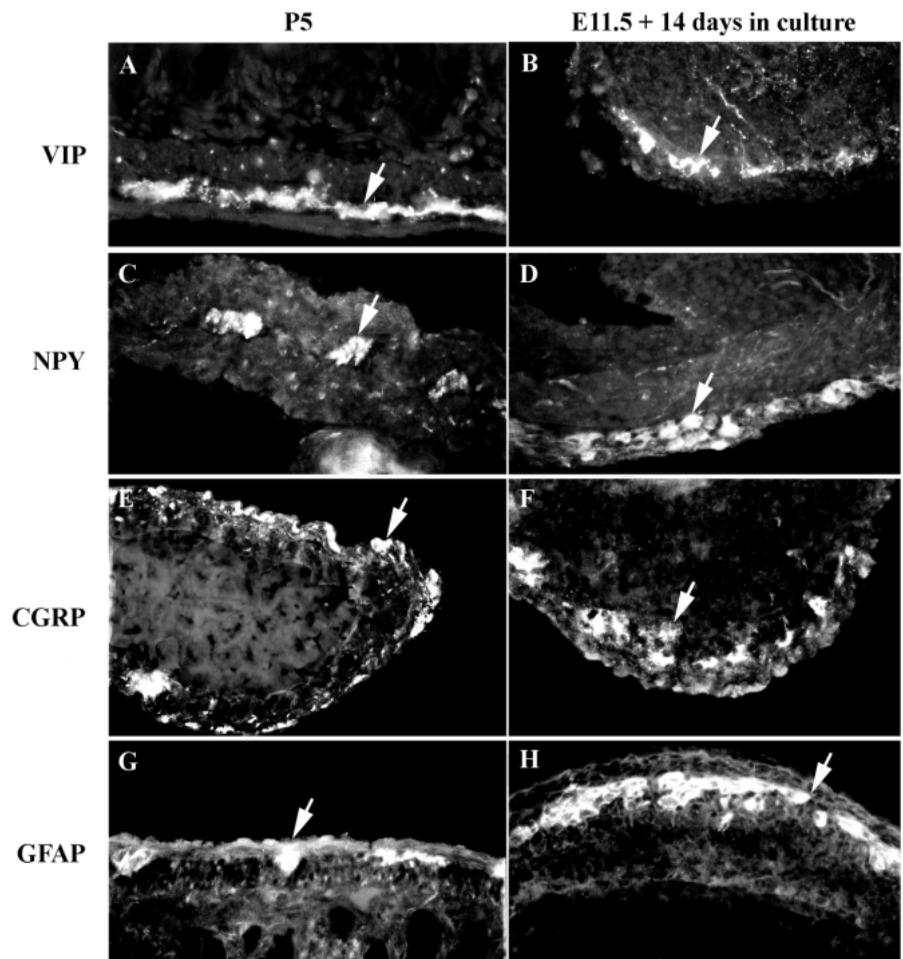


Fig. 2. Comparison of expression of neuropeptides and GFAP in gut derived from P5 mice (A,C,E,G) or from E11.5 mouse embryo gut that was cultured for 14 days (B,D,F,H). Gut sections were stained with antibodies specific for VIP (A,B), NPY (C,D), CGRP (E,F) and GFAP (G,H). Arrows indicate the immunoreactive cells.

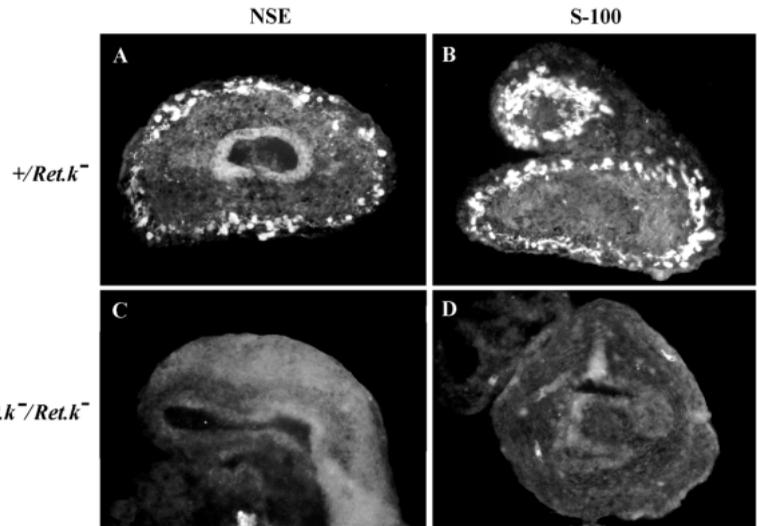


Fig. 3. RET-deficient fetal gut remains aganglionic in organ cultures. The sections shown here represent gut derived from $+/Ret.k^{-}$ (A,B) or $Ret.k^{-}/Ret.k^{-}$ (C,D) embryos which were stained with antibodies specific for NSE (A,C) or S-100 (B,D).

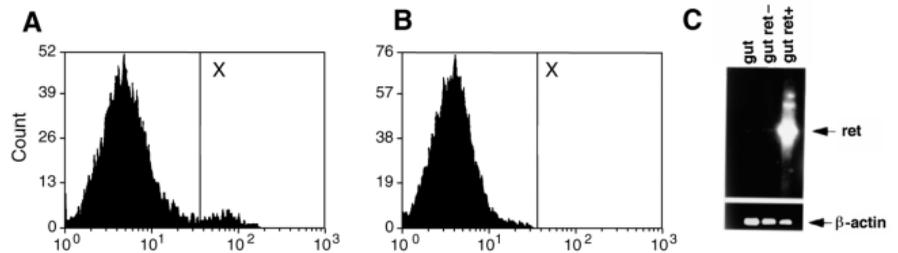
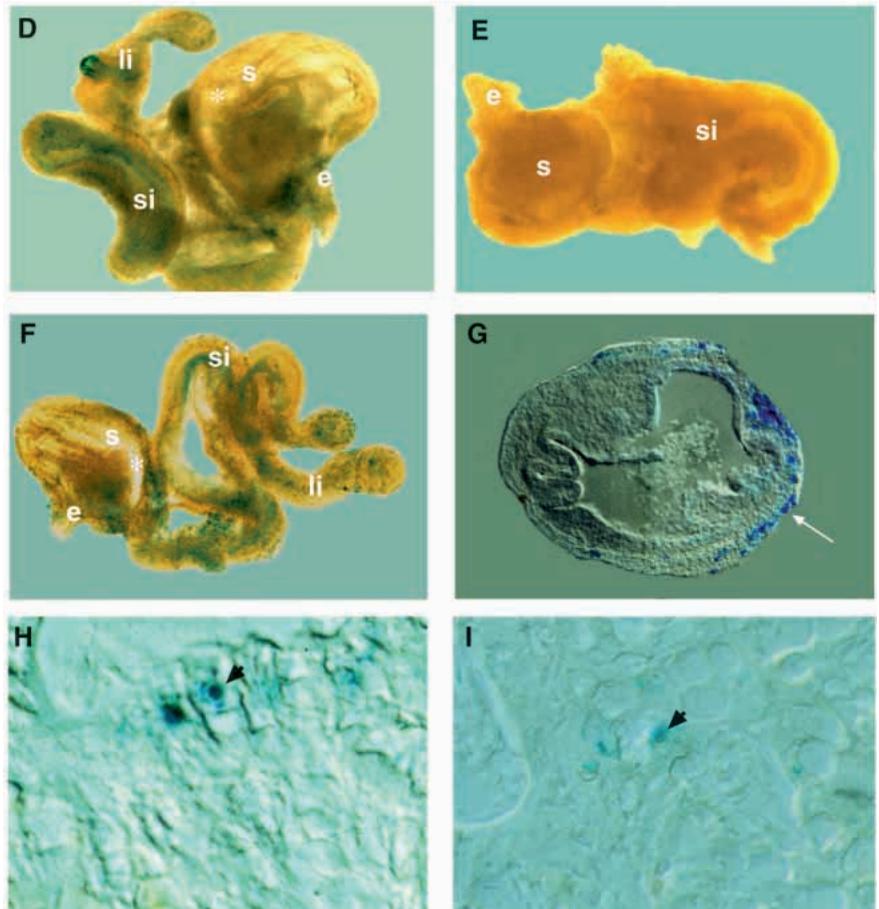


Fig. 4. RET⁺ cells from fetal gut can colonise wild-type or RET-deficient gut in organ culture. Typical FACS profile of dissociated E11.5 mouse gut following live labeling with anti-RET monoclonal antibodies (A). Cells were collected in the gated region x. (B) shows a control sort carried out in the absence of primary anti-RET antibodies. C, RT-PCR analysis of mRNA extracted from the whole population of dissociated gut cells ('gut'), non-gated cells ('gut ret⁻') or gated cells ('gut ret⁺'), using primers specific for *c-Ret* mRNA. β -actin-specific primers were used as a control for the integrity of the mRNA. The immunoselected RET⁺ cells were injected into the stomach wall of E11.5 gut derived from $+/Ret.k^{-}$ (D) or $Ret.k^{-}/Ret.k^{-}$ (F) mouse embryos. The site of injection on the stomach wall is indicated by an asterisk. A non-injected control gut is shown in panel (E). Sections of X-gal-stained guts revealed that the β -gal⁺ progeny of the RET⁺ cells (arrow) were localised at the periphery of the gut wall (G). Addition of BrdU in the culture medium resulted in incorporation of this analog in a large percentage of exogenous RET⁺ cells, as indicated by the nuclear staining of β -gal⁺ cells (arrow) upon staining with anti-BrdU antibodies (H). No such staining was observed when BrdU was omitted from the culture medium (I). e, esophagus; li, large intestine; s, stomach; si, small intestine.



and colonising enteric and non-enteric ganglia of the PNS upon transplantation along defined migratory pathways of NC in younger embryos (Rothman et al., 1990, 1993). To examine the ability of purified enteric NC to recolonise gut in vitro, we generated chimaeric guts by microinjecting genetically marked enteric NC cells into the wall of gut in organ culture. NC cells were isolated from embryos derived from PTY mice (kindly provided by Dr R. Beddington, NIMR), which express ubiquitously the bacterial *lacZ* gene, thus permitting identification of the injected NC cells and their progeny by β -galactosidase (β -gal) histochemistry. To isolate the enteric NC cells from the gut of PTY embryos, we adopted the strategy that was originally developed by Anderson and colleagues. Using a cocktail of monoclonal antibodies against the RET RTK and fluorescence-activated cell sorting (FACS), these investigators isolated a population of cells from the gut of E14.5 rat embryos that was highly enriched for NC-derived cells (Lo and Anderson, 1995). The same strategy and the same reagents (kindly provided by Dr D. Anderson, Caltech) were used to purify a population of enteric NC cells from E11.5 mouse embryo gut (Fig. 4A,B). To confirm that the immunoselected cells, which represent approximately 4-5% of the total gut cell population, represent the RET-expressing enteric NC, two types of assays were performed. First, expression of *c-Ret* mRNA was analyzed by RT-PCR in the immunoselected and unselected fractions. As shown in Fig. 4C, the cell fraction isolated on the basis of binding to anti-RET antibodies is highly enriched for *Ret* mRNA relative to its non-binding counterpart. Second, plating of the isolated RET⁺ cells resulted in cultures highly enriched in neuronal cells, as previously reported for the RET⁺ cells isolated from the gut of rat embryos (Lo and Anderson, 1995; S. Taraviras, M. G. and V. P., unpublished observations).

A relatively small number (50-100) of RET⁺ cells isolated from the gut of E11.5 PTY embryos were microinjected isochronically into the stomach wall of gut derived from Parkes embryos. Upon culturing for 7 days, β -gal histochemistry was used to reveal the exogenous cells and their progeny. As shown in Fig. 4D, a large number of β -gal⁺ cells was present in all injected guts ($n=58$) at the end of the culture period, indicating extensive proliferation of the exogenous RET⁺ cells. No β -gal⁺ cells were present in control (uninjected) guts (Fig. 4E). Further evidence supporting proliferation of the injected cells was derived from BrdU incorporation experiments. Culture of guts in medium containing BrdU resulted in incorporation of this analog into a large percentage of *lacZ*-expressing cells as revealed by double labeling using β -gal histochemistry and anti-BrdU antibodies (Fig. 4H,I). In addition to extensive proliferation, the donor RET⁺ cells were also capable of colonising the bowel uniformly along its long axis. Thus, apart from the stomach (original site of injection), β -gal⁺ cells were present in

the esophagus, small intestine and the large intestine, indicating extensive migratory capacity of the donor cells and their progeny (Fig. 4D). Furthermore, the progeny of the injected RET⁺ cells were located, along with the cells of the endogenous ENS, in the periphery of the gut wall embedded within the muscularis externa (Fig. 4G).

In the next series of experiments, we determined the ability of enteric NC to colonise RET-deficient aganglionic gut. For this, RET⁺ cells isolated from the gut of E11.5 PTY embryos were injected into the stomach wall of E11.5 gut derived from $+/Ret.k^{-}$ intercrosses and the efficiency of colonisation was determined by β -gal histochemistry at the end of the culture period (7 days). No difference was observed in the number or the pattern of distribution of the progeny of the injected cells when guts of various genotypes (i.e. $+/+$, $n=25$; $+/Ret.k^{-}$, $n=41$; $Ret.k^{-}/Ret.k^{-}$, $n=23$) were compared. An example of a chimaeric aganglionic gut generated by injection of exogenous RET⁺ cells is shown in Fig. 4F. As in wild-type guts, the progeny of the injected cells were detected throughout the length of the gut (Fig. 4D,F) and were localised at the periphery of the gut wall (data not shown). Overall, our data show that the RET⁺ enteric NC cells from midgestation mouse embryos have a high degree of proliferative and migratory capacity and are capable of colonising fetal gut wall irrespective of its genotype at the *c-Ret* locus and the presence of the intrinsic ENS.

Single RET⁺ cells can generate progeny capable of colonising wild-type and aganglionic gut in vitro

Given the potential heterogeneity of the exogenous RET⁺ cells, it was not clear from the above experiments whether all the injected cells were capable of responding to the same extent to any proliferative and migratory signals present in the mesenchyme of wild-type and RET-deficient gut. To address

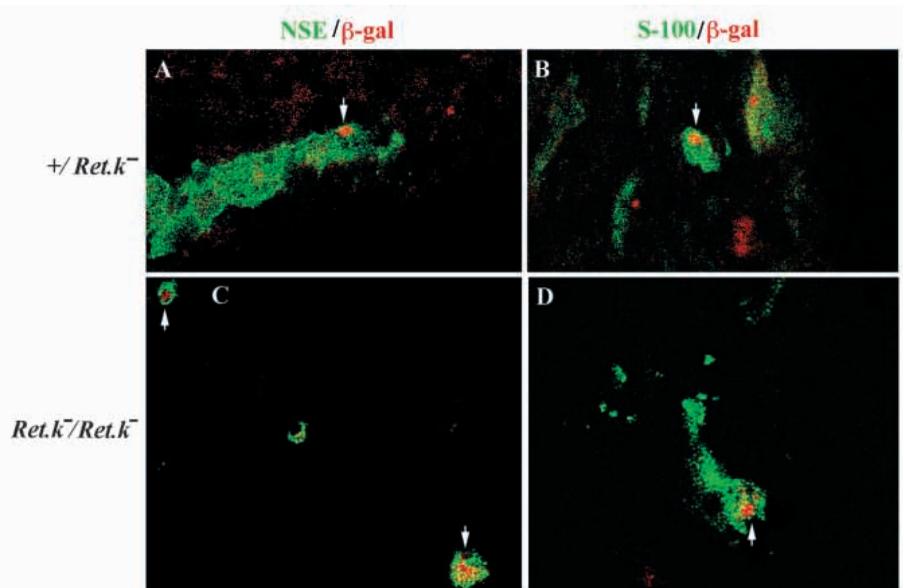


Fig. 5. RET⁺ cells isolated from E11.5 mouse gut can differentiate into mature neurons and glia upon reintroduction into $+/Ret.k^{-}$ (A,B) or $Ret.k^{-}/Ret.k^{-}$ (C,D) bowel in explant culture. Sections were double stained with antibodies specific for β -gal (all panels) and NSE (A,C) or S-100 (B,D). Arrows indicate the β -gal-specific signal surrounded in each case by NSE or S-100-specific signal.

this issue, single RET⁺ cells of PTY origin were introduced into the stomach wall of gut derived from E11.5 embryos of +/Ret.k⁻ intercrosses. At the end of a 7-day culture period, β -gal histochemistry was used to reveal the progeny of the injected cell and their location in the chimaeric gut. In all cases analysed ($n=52$), the injected RET⁺ cell had proliferated and generated a large number of progeny (>100), which either formed a cohort of cells at various distances from the site of injection or were distributed uniformly throughout the organ, colonising the esophagus, stomach and intestines (Fig. 6A,B and data not shown). Interestingly, despite representation of all genotypes expected from Mendelian segregation (i.e. +/+, +/Ret.k⁻ and Ret.k⁻/Ret.k⁻), no obvious correlation could be made between the number and distribution of progeny of the injected cell and the genotype of the recipient gut (Fig. 6A,B; and data not shown). These data suggest that a large majority of single RET⁺ cells introduced into the foregut of E11.5 bowel were capable of responding to migratory and proliferative signals that were likely to be present in fetal gut and that such signals were also present in the mesenchyme of aganglionic RET-deficient gut.

Developmental potential of RET⁺ fetal gut cells

In order to study the developmental potential of enteric NC cells, we initially analysed the capacity of a population of RET⁺ cells to generate the neuronal and glial cell lineages of the ENS upon introduction into fetal gut in organ culture. For this, approximately 50-100 RET⁺ cells isolated from E11.5 PTY mouse gut were injected isochronically into the stomach wall of gut isolated from Parkes embryos. At the end of the culture period (7 days), serial sections of the recipient gut were double-labeled with antibodies specific for neuronal or glial lineage markers (NSE or S-100, respectively) and β -galactosidase and analysed using confocal laser microscopy. In all cases examined ($n=20$), we identified large numbers of β -gal⁺ cells that also expressed one of the lineage markers (Fig. 5A,B). Based on the relative proportions of the neuronal and glial components of the β -gal⁺ cell population, we estimated that the two cell lineages were generated with approximately equal efficiency.

In the next series of experiments, wild-type RET⁺ cells were microinjected into guts derived from embryos of +/Ret.k⁻ intercrosses and their progeny were analysed for co-expression of β -gal and the neuronal and glial cell lineage markers. Our data show that these cells were capable of generating both cell lineages of the ENS irrespective of the genotype (+/+; $n=53$, +/Ret.k⁻; $n=85$ or Ret.k⁻/Ret.k⁻; $n=80$) of the recipient gut mesenchyme (Fig. 5C,D). We conclude that RET⁺ cells can differentiate into mature neurons and glia within the microenvironment of RET-deficient gut in the absence of endogenous ENS.

Developmental potential of single RET⁺ cells

In order to examine to what degree the RET⁺ cell population consists of a mixture of heterogeneous progenitors irreversibly committed to neuronal or non-neuronal cell fates, single RET⁺ cells isolated from the gut of E11.5 PTY embryos, were microinjected isochronically into the stomach wall of fetal gut (from Parkes embryos). Upon culturing of the recipient gut for 7 days, co-expression of β -gal and lineage markers (NSE or S-100) was examined in the progeny of the injected cell. Our data

show that, in all cases examined ($n=76$), single RET⁺ cells were capable of generating approximately equal numbers of mature neurons and glial cells (Fig. 6C,D), suggesting that the population of RET⁺ cells from E11.5 mouse embryo gut consists of multipotential progenitors that are capable, under the appropriate conditions, of generating both cell lineages of the ENS.

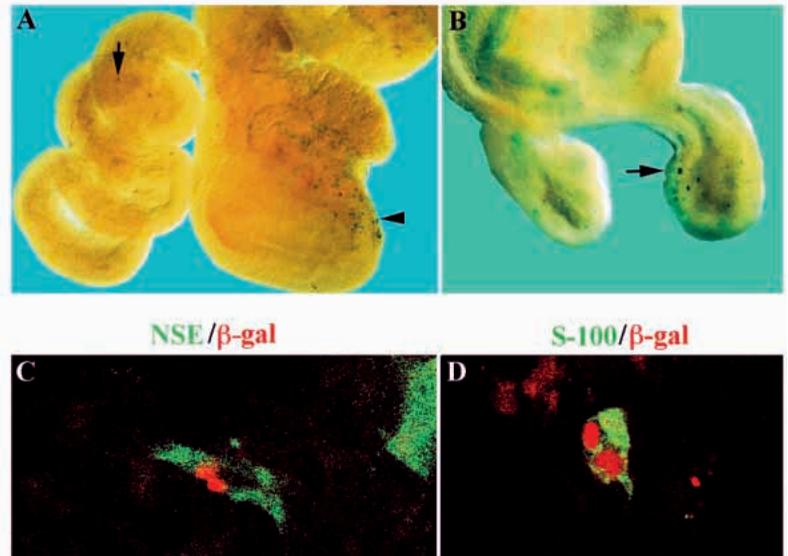
DISCUSSION

Organ culture of mouse fetal gut

We have developed an organ culture system of mouse fetal gut which supports differentiation and morphogenesis of the intrinsic ENS. This system consists of the entire bowel dissected from midgestation (E11.5) mouse embryos and maintained in a collagen gel matrix or as a free-floating organ in defined medium. At the time of dissection, the gut contains mainly undifferentiated NC-derivatives, but subsequent in vitro culture results in the differentiation of mature neurons and glia which share at least some of the phenotypic characteristics of their counterparts in vivo. In addition to the intrinsic ENS precursors, this organ culture system can also support migration, proliferation and differentiation of a phenotypically defined population of enteric NC cells (those expressing the RET RTK) upon reintroduction into the gut wall.

The ENS of vertebrates is part of the PNS (Le Douarin, 1982). However, many of its features, such as the vast number and diversity of neurons and glia, the histological organisation of the enteric ganglia and the formation of highly complex local reflex circuits that control motility and secretions independently of the CNS, distinguish it from other simpler parts of the PNS (Gershon et al., 1994). Despite its complex organisation, the ENS is derived, as is the rest of the PNS, from the NC (Durbec et al., 1996; Le Douarin and Teillet, 1973; Yntema and Hammond, 1954). Moreover, molecules known to have a critical function in autonomic and sensory neurogenesis in the PNS, are also likely to be important for the development of the ENS (Gershon, 1997). Although significant progress has been made towards the identification of genetic loci that are important for ENS development in mammals, the cellular and molecular mechanisms controlling ENS histogenesis are less well understood compared to other parts of the PNS. Some of the reasons responsible for this relative paucity of information have been (1) the low percentage of ENS precursors relative to the total cellular population of fetal gut, which prohibited their efficient purification, (2) the absence of in vitro experimental systems that allowed ENS histogenesis to proceed under conditions that mimicked the normal cellular interactions observed in vivo, and (3) the lack of molecular markers that uniquely define neuronal or glial ENS identity. As a result, such identity needs to be defined by a combination of multiple parameters, including combinatorial gene expression, functional diversification of cells, position in the gut wall and synaptic connectivity, which can only be realised and studied in whole organ systems (Furness and Costa, 1987; Gershon et al., 1994). Recent advances have addressed some of the problems associated with the study of the vertebrate ENS. Thus, using antibodies specific for cell surface molecules expressed in enteric NC cells (NC1, HNK1 and RET), several groups have immunoselected subpopulations of ENS

Fig. 6. Proliferation and differentiation of single RET⁺ cells introduced into the stomach wall of wild-type gut cultures. (A,B) show whole-mount preparations of chimaeric guts generated by microinjection of single RET⁺ cells into the stomach wall and stained at the end of a 7-day culture period with X-gal. In the gut shown in A a group of progeny of the injected cell (arrowhead) have remained near the injection site in the stomach. Other progeny have migrated into the small intestine (arrow). In the gut shown in B most of the progeny have migrated into various regions of the gut, including the cecum (arrow). In C,D sections of similarly generated chimaeric guts were double stained for antibodies specific for β -gal and NSE (C) or S-100 (D). In both cases the two signals were clearly co-localised within the same cell.



precursors and studied their commitment and differentiation in various cell culture assays (Chalazonitis et al., 1994; Hearn et al., 1998; Lo and Anderson, 1995). In addition to these reports, several investigators have used organotypic cultures of fetal gut in order to study the colonisation of the bowel by NC and various aspects of ENS cell differentiation (Dreyfus et al., 1977; Jacobs-Cohen et al., 1987; Rothman and Gershon, 1982; Rothman et al., 1984). However, in these studies the gut tissue attached to the culture substratum and both mesodermal and NC-derived cells migrated extensively, thus resulting in loss of the three-dimensional organisation of the gut and drastic alteration of the normal cellular microenvironment. Finally, aspects of ENS development have been studied in intact segments of fetal gut transplanted under the kidney capsule of adult mice (Kapur et al., 1992; Young et al., 1996, 1998). However, despite the preservation of the three-dimensional organisation of the gut wall, such transplants are relatively inaccessible for experimental manipulations and continuous monitoring. The experimental system that we have developed and used here has several advantages relative to existing systems: (1) it maintains the integrity and overall organisation of the bowel and thus allows the study of the developing ENS under conditions in which normal cellular interactions are preserved, (2) in combination with the isolation of genetically marked populations of enteric NC cells from fetal bowel and their reintroduction into the gut wall in vitro, it allows the efficient generation of large numbers of chimaeric organs and thus the detailed study of interactions between the exogenous cells and the recipient mesenchyme and (3) the use of serum-free culture conditions and the availability of a variety of mutant mouse strains, allows the in vitro analysis of the response of defined populations of intrinsic or extrinsic populations of ENS progenitors to various growth factors (supplemented to the culture medium) in conjunction with the genotype of the recipient gut.

RET⁺ cells can colonise aganglionic gut in organ culture

In our initial experiments, we used the fetal gut culture system to address questions relating to the cellular mechanisms of

action of *c-Ret*. Genetic studies in humans and mice have shown that the RET RTK has a critical role in the development of the ENS (Robertson et al., 1997). Despite the established role of this receptor in gut neurogenesis, the primary cell type in which it functions and its mechanism of action remain largely unknown. For example, it is presently unclear whether the intestinal aganglionosis of RET-deficient embryos is due to a cell autonomous requirement of *c-Ret* in all NC derivatives that fail to develop in the gut of these embryos. An alternative interpretation would be that RET is required only in a small fraction of RET-expressing cells, the absence of which conditions the gut mesenchyme in such a way that becomes unable to support further colonisation by other NC cells. Our data indicate that wild-type RET⁺ cells are capable of colonising the wall of wild-type and aganglionic (RET-deficient) gut with equal efficiencies and that this cell population is relatively homogeneous in its response to signals from the recipient (wild-type or mutant) mesenchyme. Overall, these findings, together with the established expression of *c-Ret* in the NC-derived component of the fetal gut, suggest strongly that the RET receptor functions cell autonomously in the enteric NC. Moreover, our findings show that aganglionic gut mesenchyme derived from *Ret.k*⁻ homozygous embryos produces all the necessary signals required for its colonisation by wild-type RET⁺ cells.

RET⁺ cells from fetal gut are migratory

In vivo grafting experiments, in which segments of gut from quail embryo were backtransplanted into the pathway of NC migration in chicken embryos, showed that NC-derived cells that had already colonised the bowel, reinitiated migration and colonised peripheral ganglia (including enteric ganglia) at various distances from the grafting site (Rothman et al., 1990, 1993). However, in these experiments, no attempt was made to attribute the migratory properties of the NC-derivatives to a particular cellular population. Expression of *c-Ret* in the PNS of vertebrate embryos is mainly associated with postmigratory neurons. However, *c-Ret* mRNA has also been detected in subpopulations of migrating cranial NC cells, such as the neurogenic crest of the prospective VIIth cranial ganglion and

the enteric NC throughout bowel colonisation (Pachnis et al., 1993; Robertson and Mason, 1995; Schuchardt et al., 1995; Tszuzuki et al., 1995). Despite these observations, no direct association has been made in vivo between expression of *c-Ret* and the migratory potential of any NC-derived cell type. Furthermore, in vitro cultures of RET⁺ cells isolated from the gut of E14.5 rat embryos failed to reveal any migratory potential of these cells (Lo and Anderson, 1995). Results presented here show that the majority of RET⁺ cells introduced into the wall of fetal gut in organ culture show extensive migratory capacity. That such migration is unlikely to result from passive transport of the injected cells by the movements of the recipient cell population, is suggested by control experiments in which non-NC-derived cells of fetal gut (i.e. RET⁻) introduced into the gut wall, responded dramatically differently to their RET⁺ counterparts by showing minimal migratory behaviour (data not shown).

A number of studies have shown that, during colonisation of the bowel, the enteric NC migrates in a rostrocaudal direction (Kapur et al., 1992; Pachnis et al., 1993; Young et al., 1998). The source and identity of signals that control the initial entry of NC cells into the foregut mesenchyme and their subsequent migration within the gut wall are currently unknown. Generally, in our experiments, the injected RET⁺ cells reproduced the normal migratory behaviour, traveling from the stomach towards the large intestine. However, the presence of RET⁺ cell progeny in the esophagus of the recipient gut as well, indicates that a proportion of the injected cells followed a 'reverse' migratory pathway (stomach to esophagus) (Fig. 4D,F), which has not been previously observed in the bowel of mammalian embryos. The reasons for this 'abnormal' migratory behaviour are not known. Since the injection experiments have been performed at a stage (E11.5) at which the proximal bowel has already been colonised by NC, these findings could suggest that the signals responsible for the directed migration of enteric NC in a given segment of the fetal bowel are transient and disappear upon completion of the colonisation of that segment. However, an alternative interpretation of these observations is based on the suggestion that 'population pressure' forces the enteric NC to always move away from the point of entry into the bowel (Hearn et al., 1998). Thus, in vivo, entry of the enteric NC at the foregut results inevitably in their migration towards the hindgut while, in our in vitro microinjection experiments, introduction of cells in the stomach wall leads to symmetric migration away from the injection site, both anteriorly and posteriorly.

In addition to the migration of RET⁺ cells along the long axis of the gut, our experiments show that the majority of the progeny of the injected cells position themselves in the outer smooth muscle layer and apparently integrate with the ENS that develops from the endogenous NC cells. Although the molecular mechanism(s) responsible for the stereotypic ring-like organisation of the ENS at the periphery of the gut wall are currently unknown, this organisation is likely to result from direct interaction between the enteric NC cells and the mesenchyme-derived smooth muscle cells developing at the periphery of the gut wall (C. V. M-G. and V. P.; unpublished observations). This is further supported by our findings, which show that RET⁺ cells microinjected into *Ret.k⁻/Ret.k⁻* gut cultures position themselves appropriately at the periphery of the gut wall. Overall, our experiments suggest that the signals

that are necessary for migration of enteric NC and their homing to the appropriate locations are present in wild-type and RET-deficient aganglionic gut developing in organ culture. The combination of the gut culture system described here and vital labeling of RET⁺ cells will allow the detailed analysis of the migratory behaviour of individual NC-derived cells over a period of time and under a variety of conditions. In addition to the identification of the molecular signals responsible for enteric NC cell migration, it will be interesting to determine to what extent the fetal gut cultures can also support migration of other non-enteric cell types. The availability of an experimental system that can support migration of diverse types of cells could provide answers to a number of critical questions relating to cell migration during vertebrate embryogenesis.

RET⁺ cells from E11.5 mouse gut are multipotential progenitors with a high degree of proliferative capacity

The data presented here show that, in addition to their migratory capacity, RET⁺ cells isolated from E11.5 mouse gut retain a high degree of proliferative potential; both, a small population of cells as well as single cells introduced into the gut wall, were capable of dividing and generating large numbers of progeny. In the case of the single cell experiments, the number of progeny generated by the exogenous cells, albeit generally large, was variable with some clones containing as few as 100 and others as high as 1000 cells. These data suggest that the RET⁺ cell population is somewhat heterogeneous with regard to its proliferative ability. In addition to their extensive proliferative capacity, RET⁺ cells isolated from fetal gut constitute a developmentally homogeneous population of cells. A relatively small group of cells, as well as single randomly selected RET⁺ cells introduced into the stomach wall of gut cultures were capable of generating mature neurons and glia. With regard to the developmental potential of single cells, our conclusions thus far are based on injections of RET⁺ cells into wild-type gut. However, a small series of similar single-cell injection experiments have also been performed in gut derived from *Ret.k⁻/Ret.k⁻* embryos. Surprisingly, despite extensive proliferation of the injected cell (see Results section) and the identification of large numbers of S-100-expressing cells among its progeny, we failed to detect any NSE + derivatives (our preliminary studies). Although the full developmental potential of individual RET⁺ cells is convincingly established by the single-cell injections into wild-type gut, the complete understanding of the mechanisms by which similar injections into RET-deficient gut fail to generate any neurons would require more extensive experimental analysis.

Anderson and colleagues have previously studied the developmental potential of RET⁺ cells isolated from the gut of rat embryos and showed that, depending on the culture conditions, they were capable of generating neuronal and non-neuronal phenotypes. However, in addition to this multipotential subpopulation, a sizable fraction of cells (up to 50%) showed restricted proliferative potential and was irreversibly committed to the neuronal cell fate (Lo and Anderson, 1995; Lo et al., 1997). Similar restriction was also observed in clonal NC cultures established from the gizzard of E4-8 chick embryos (Sextier-Sainte-Claire Deville et al., 1994). The reason for the apparent difference between these cell culture studies and the data presented here, is not clear.

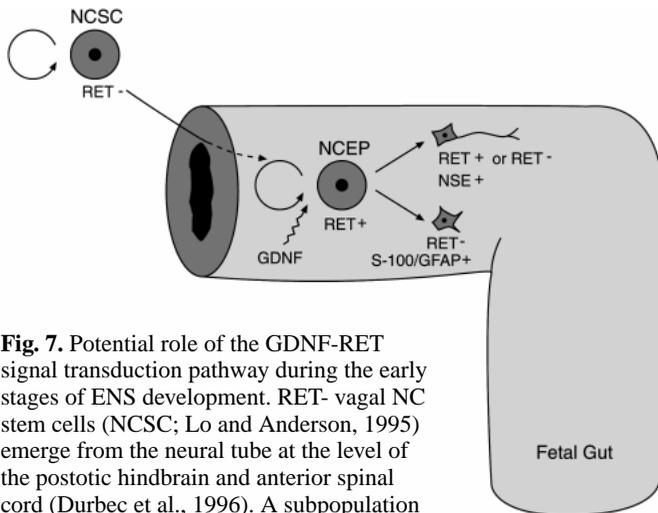


Fig. 7. Potential role of the GDNF-RET signal transduction pathway during the early stages of ENS development. RET⁻ vagal NC stem cells (NCSC; Lo and Anderson, 1995) emerge from the neural tube at the level of the postotic hindbrain and anterior spinal cord (Durbec et al., 1996). A subpopulation of these cells initiate expression of *c-Ret* prior to entry into the gut and form the RET⁺ NC-derived ENS progenitors (NCEP). Within the gut microenvironment and under the influence of GDNF, NCEP cells maintain their multipotential capacity, proliferate extensively and colonise the entire bowel. Downregulation of *c-Ret* expression is associated with differentiation of the glial cell lineage. Similarly, such downregulation might also be important for differentiation of a subset of enteric neurons. In addition to this early effect on NCEP cells, the RET RTK could function in the differentiation of the RET⁺ subpopulation of neuronal cells during later stages of ENS development.

Regarding the study of Lo and Anderson, a potential explanation could be that the bowel of E11.5 mouse embryos, being slightly younger compared to that of E14.5 rat embryos, contains NC cells at an earlier developmental stage. However, this seems unlikely since the vast majority of RET⁺ cells isolated from the gut of E12.5 mouse embryos (a stage equivalent to E14.5 rat embryos) behaved identically to their earlier counterparts (D. N. and V. P., unpublished data). A direct comparison between our studies and those of Sextier-Sainte-Claire Deville et al. (1994) is more difficult given potential differences in the development of the avian and mammalian ENS. However, a hypothesis that could reconcile our results and the *in vitro* studies is that the integrity of the gut wall, as maintained under our organ culture conditions, generates a cellular microenvironment that is necessary and sufficient for RET⁺ cells to retain their multipotential and highly proliferative status; removal of these cells from the gut microenvironment, prevents proliferation and progressively induces their premature and irreversible commitment to certain cellular phenotypes (Fig. 7).

The signals that maintain the RET⁺ cells in the state of proliferating multipotential progenitors are likely to be derived from the gut wall mesenchyme since no difference was detected in the response of these cells upon introduction into wild-type or RET-deficient aganglionic gut. Although the identity of such signals has not been established, it is likely that the GDNF-RET signal transduction pathway has an important role in the 'progenitor maintenance' function of the gut wall in organ culture. First, the RET signalling pathway is likely to be activated under our organ culture conditions, since high levels of expression of the RET receptor, the co-receptor GFR α 1 and GDNF are preserved with a temporal and cell-specific pattern

similar to that observed *in vivo* (D. N. and V. P., unpublished data). In addition, recent findings have shown that GDNF, a molecule expressed by the mesenchyme of the gut wall (Hellmich et al., 1996; Suvanto et al., 1996), has a clear proliferative effect on RET⁺ cells of mammalian and avian gut *in vitro* (Hearn et al., 1998) and our unpublished data). Finally, superactivation of the RET RTK by exogenous GDNF added to the organ culture medium, increases dramatically the number of both neurons and glia, suggesting that this neurotrophic factor promotes the proliferation of uncommitted multipotential progenitors (D. N. and V. P.; unpublished observations). Based on this, a prediction emerges that downregulation of expression of *c-Ret* and *Gdnf* is necessary for normal differentiation of the ENS precursors. In this respect, it is interesting that during ENS development, expression of *c-Ret* is switched off in differentiating glial cells and in a significant proportion of the neuronal cells ((Durbec et al., 1996; Pachnis et al., 1993; Tsuzuki et al., 1995) and our unpublished observations), while expression of *Gdnf* is also downregulated in the surrounding mesenchymal smooth muscle layers (Moore et al., 1996). Ectopic expression of a constitutively active form of RET in isolated enteric NC cells prior to their introduction into guts in culture, should allow us to test this prediction.

The significance of such a 'progenitor maintenance' function of the GDNF-RET signal transduction pathway would be self evident given the demanding task of a relatively small group of NC cells that enter the foregut to populate a relatively large and rapidly growing bowel over a period of several days and generate large numbers of neurons and glia. Furthermore, such function could explain the phenotype observed in patients with Hirschsprung's disease in which mutations at the *c-RET* locus could lead to premature commitment and terminal differentiation of the ENS progenitors, prior to colonisation of the entire gut. Of course such an early function of the RET receptor does not exclude additional roles of this molecule at later stages of embryogenesis relating to differentiation and morphogenesis in the ENS. Experiments are in progress to address such additional functions using the gut explant system reported here.

Most of the cases of Hirschsprung's disease are treated at birth by surgical removal of the aganglionic segment of the colon. Such an approach, albeit generally effective, is of course not feasible in cases of extented or total intestinal aganglionosis. The ability of a phenotypically defined population of enteric NC cells to colonise the mesenchyme of aganglionic gut and differentiate into mature neurons and glia suggests the feasibility of alternative therapeutic approaches for the treatment of severe cases of Hirschsprung's disease which are based on transplantation of ENS progenitors. In this regard, it is critical to study in further detail the RET⁺ enteric NC cells, as well as other defined populations of progenitors from the PNS and CNS of mammalian embryos, as a potential source of progenitors that are capable of colonising aganglionic bowel *in vitro* and *in vivo*.

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