

# Neuron and glia generating progenitors of the mammalian enteric nervous system isolated from foetal and postnatal gut cultures

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## Summary

Cultures of dissociated foetal and postnatal mouse gut gave rise to neurosphere-like bodies, which contained large numbers of mature neurons and glial cells. In addition to differentiated cells, neurosphere-like bodies included proliferating progenitors which, when cultured at clonal densities, gave rise to colonies containing many of the neuronal subtypes and glial cells present in the mammalian enteric nervous system. These progenitors were also capable of colonising wild-type and aganglionic gut in organ culture and had the potential to generate differentiated progeny that localised within the intrinsic ganglionic plexus. Similar progenitors were also derived from the normoganglionic small intestine of mice with

colonic aganglionosis. Our findings establish the feasibility of expanding and isolating early progenitors of the enteric nervous system based on their ability to form distinct neurogenic and gliogenic structures in culture. Furthermore, these experiments provide the rationale for the development of novel approaches to the treatment of congenital megacolon (Hirschsprung's disease) based on the colonisation of the aganglionic gut with progenitors derived from normoganglionic bowel segments.

Key words: Enteric nervous system, Neural crest progenitors, Hirschsprung's disease

## Introduction

The enteric nervous system (ENS) is the part of the peripheral nervous system (PNS) that controls the peristaltic and secretory activity of the gut wall. It is composed of a large number of neurons and glia, which are organised into interconnected ganglia distributed throughout the length of the gut (Gershon et al., 1994; Le Douarin, 1999). The majority of neurons and glia of the ENS are derived during embryogenesis from the vagal neural crest (Durbec et al., 1996; Le Douarin and Teillet, 1973; Yntema and Hammond, 1954). These pre-enteric neural crest cells (PENCCs) invade the foregut mesenchyme (E9.5-10.0, called thereafter enteric neural crest cells-ENCCs), and migrating in a rostrocaudal direction colonise the entire length of the gut over a period of 4 days (E9.5-13.5) (Durbec et al., 1996; Kapur et al., 1992; Young et al., 1998). Failure of complete colonisation of the gut by ENCCs results in absence of enteric ganglia (aganglionosis), usually in the colon, leading to peristaltic misregulation and severe intestinal obstruction [Hirschsprung's disease (HSCR), congenital megacolon] (Chakravarti, 2001).

HSCR is the most common neurocristopathy in humans affecting 1:4500 newborns (Chakravarti, 2001; Swenson, 2002). It appears either sporadically or has a familial basis often associated with other developmental defects. The main form of treatment of HSCR is surgical resection of the aganglionic bowel and anastomosis of the remaining gut segments, a procedure aimed at relieving the life threatening

consequences of obstruction, and restoring bowel movements (Swenson, 2002). However, the outcome of this approach is often unsatisfactory as it is frequently associated with short-term postoperative complications or failure to restore bowel function in the long-term (Tsuji et al., 1999). The better molecular and cellular understanding of HSCR pathogenesis and the identification of genes that play an important role in enteric neurogenesis in mammals offers new opportunities for the development of therapies based on gene or cell replacement strategies.

Among the genes that have been identified as critical players in ENS development is *Ret* which encodes the receptor tyrosine kinase (RTK) RET, the main signalling component of cell surface multisubunit receptors for glial cell line-derived neurotrophic factor (GDNF) and other members of the GDNF family of ligands (Baloh et al., 2000; Saarma, 2000). Mutations in *RET* account for approximately 50% of familial HSCR cases (Chakravarti, 2001) and mice with null mutations in either *Ret* (*Ret<sup>k-</sup>*) or *Gdnf* (*Gdnf<sup>-</sup>*) have complete intestinal aganglionosis (Durbec et al., 1996; Enomoto et al., 2001; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Schuchardt et al., 1994). *Ret* encodes two isoforms, RET9 and RET51, which differ in their carboxy-terminal sequences (Tahira et al., 1990). Analysis of the effects of monoisofomeric alleles of *Ret*, which encode either RET9 (*miRet<sup>9</sup>*) or RET51 (*miRet<sup>51</sup>*), demonstrated that RET9 is sufficient to support normal development of the ENS and that expression of RET51 in the

absence of RET9 is associated with failure of enteric ganglia formation in the distal two thirds of the colon (de Graaff et al., 2001).

Several transcription factors have also been identified as important regulators of ENS development. Among them is SOX10, a member of the high mobility group (HMG) family of proteins, which include the SRY testis-determining factor and other members of the SOX subfamily (Kuhlbrodt et al., 1998; Wegner, 1999). The critical role of SOX10 in PNS development is highlighted by the severe defects of sensory, autonomic and enteric ganglia in mice lacking SOX10 (Britsch et al., 2001; Herbarth et al., 1998; Kapur, 1999; Paratore et al., 2001; Southard-Smith et al., 1998). In addition, humans and mice heterozygous for mutations in this locus have distal colonic aganglionosis (Herbarth et al., 1998; Parisi and Kapur, 2000; Pingault et al., 1998; Southard-Smith et al., 1998). Defects in ENS development have also been identified in mice homozygous for a null mutation of *Mash1* (*Ascl1* – Mouse Genome Informatics) a locus encoding a basic helix-loop-helix (bHLH) proneural factor (Johnson et al., 1990). MASH1-deficient embryos have a defect in gut colonisation by neural crest cells, while mutant newborn animals lack specific subsets of enteric neurons (Blaugrund et al., 1996).

Enteric neurogenesis is associated with a highly regulated programme of gene expression that defines distinct stages of cell commitment and differentiation. Thus, during the early stages of migration PENCCs express *Sox10*, which is important for the maintenance of the undifferentiated multipotential state of ENS progenitors, but are negative for MASH1 or RET (SOX10+/MASH1-/RET-) (Durbec et al., 1996; Kim et al., 2003; Paratore et al., 2002). However, as PENCCs arrive in the vicinity of the foregut, they begin to express *Mash1* and *Ret* (SOX10+/MASH1+/RET+) (Blaugrund et al., 1996; Durbec et al., 1996). Induction of *Mash1* is likely to be associated with neuronal specification (Lo and Anderson, 1995; Lo et al., 1997) while RET at this stage is required for the survival, proliferation, differentiation and migration of ENCCs (Gianino et al., 2003; Taraviras et al., 1999). Terminal differentiation of subsets of committed neurogenic progenitors (indicated by expression of neuron-specific tubulin-TuJ1 and PGP9.5) takes place from the earliest stages of gut colonisation and continues for at least two weeks after birth.

Progenitors of the ENS have been isolated from rodent embryos and partially characterised in culture. Using antibodies against RET and fluorescence-activated cell sorting (FACS), Anderson and colleagues isolated from the gut of rat embryos, a population of cells which in clonogenic assays generated mostly neurons (Lo and Anderson, 1995). However, the equivalent murine cell population when grafted into foetal gut maintained in organotypic culture generated both neuronal and glial progeny (Natarajan et al., 1999). More recently, a self-renewing population of neural crest stem cells (NCSCs) has been isolated from the gut of foetal and postnatal rats using antibodies against cell surface markers and flow cytometry (Bixby et al., 2002; Kruger et al., 2002). In vitro clonogenic assays and in vivo engraftment of such gut-derived NCSCs showed that they are capable of generating both neuronal and glial cells. However, the ability of these cells to re-colonise gut and differentiate within its wall has not been established.

Isolation of ENS progenitors from fresh tissue so far has relied

on the use of cell surface markers, an approach that generally requires relatively large amounts of starting material. Here, we have used cultures of dissociated gut and retrovirus-mediated gene transfer to isolate multipotential progenitors of enteric neurons and glia from both foetal and postnatal gut at least up to 2 weeks after birth. We describe the phenotypic characteristics of these cells, their progressive differentiation in vitro and their ability to generate both neuronal and glial progeny upon transplantation into foetal gut maintained in organ culture. Finally, we show that similar progenitors can be isolated from the normoganglionic gut segments of mice with colonic aganglionosis. We discuss the implications of our findings for the treatment of HSCR by cell replacement strategies.

## Materials and methods

### Animals

Wild-type whole foetal gut and postnatal gut peels were isolated from Parkes (outbred) mice. Mutant guts were isolated from either *Ret<sup>k-</sup>* or *miRet<sup>51</sup>* homozygous animals (de Graaff et al., 2001; Schuchardt et al., 1994). The day of vaginal plug detection was considered to be E0.5.

### Isolation of enteric nervous system progenitor cells (EPCs) from foetal and postnatal gut

To generate neurosphere-like bodies (NLBs) from foetal gut tissue, whole gut was dissected from E11.5 embryos (wild-type or from *Ret<sup>k-</sup>* intercrosses) in L15 medium (Invitrogen, UK), washed with Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free PBS (Invitrogen, UK) and digested for 6 minutes with a mixture of 1 mg/ml dispase/collagenase (Roche, UK) at room temperature (RT). Dissociated tissue was washed sequentially with 1× PBS and NCSC culture medium (Morrison et al., 1999), which included 15% chicken embryo extract (CEE) and basic fibroblast growth factor (bFGF; 20 ng/ml; R&D Systems), and plated onto tissue culture dishes coated with 20 µg/ml fibronectin (Sigma). Cultures were re-fed every 2 days. Once NLBs appeared, human recombinant epidermal growth factor (hrEGF) (20 ng/ml; Calbiochem) was added to the medium.

To generate postnatal NLBs, small intestines from P2-P14 wild-type (Parkes) or *miRet<sup>51</sup>* animals were removed and cleaned. Using pairs of forceps, the outer smooth muscle layers along with the myenteric plexus were peeled off from the underlying tissue. These strips were washed in 1× PBS and treated with 1 mg/ml collagenase (Sigma) for 45 minutes at 37°C. The resulting cell suspension was washed in NCSC medium and plated on fibronectin-coated six-well dishes (NUNC). A similar protocol was used to generate NLBs from newborn *Ret<sup>k-</sup>* homozygous animals. Foetal or postnatal NLBs were either cultured for an additional 5-10 days to isolate enteric nervous system progenitor cells (EPCs) or dissociated and re-plated at low density to generate secondary or tertiary NLBs.

To isolate EPCs, NLBs were trypsinised at RT, washed with NCSC medium and passed through a mesh (pore size 50 µm) to produce a near single cell suspension, which was plated as before. Cells were infected using a mixture of NCSC medium and a GFP-expressing retrovirus suspension (1:1) in the presence of polybrene (5 µg/ml). GFP-expressing cells were isolated 24 hours later by fluorescence activated cell sorting (FACS) and plated in complete NCSC medium at a density of 50-100 cells per Sonicseal™ well and cultured for up to 15 days. Human recombinant (hr) GDNF protein (Peprotech) was used at 10 ng/ml either in complete medium or in NCSC medium lacking CEE, EGF or FGF.

Manually cut pieces of NLBs and isolated EPCs were grafted into organotypically cultured E11.5 mouse embryo gut as described previously (Natarajan et al., 1999). Detailed protocols of all the procedures described here are available upon request.

### Retrovirus production

BOSC cells were transfected with the GFP-expressing retroviral vector pMX (Kitamura, 1998), using  $\text{CaCl}_2$  and 25  $\mu\text{M}$  chloroquine for 10 hours. Cells were washed, allowed to recover and viral particles collected after 36 hours in DMEM medium plus 10% foetal calf serum, 100 U/ml antibiotic mixture, 1 mM L-glutamine (2.5 ml/10 cm plate). Viral stocks were filtered (0.45  $\mu$ ) and stored at  $-80^\circ\text{C}$ .

### Immunostaining

Cultures were fixed in 4% PFA (in 1 $\times$  PBS) for 10 minutes at RT. After washing twice in PBS + 0.1% Triton X-100 (PBT), they were incubated with blocking solution (PBT + 1% BSA + 0.15% glycine) at 4°C (overnight) or at RT (for 2-3 hours). Primary antibodies were diluted in blocking solution as follows: TuJ1(mouse; Babco, UK) 1:1000, PGP9.5 (rabbit; Biogenesis, UK) 1:400, vasointestinal peptide, VIP (rabbit; Biogenesis, UK) 1:500, neuropeptide Y, NPY (rabbit; Biogenesis, UK) 1:100, CGRP (rabbit; Biogenesis, UK) 1:100, tyrosine hydroxylase, TH (rabbit; Chemicon, UK) 1:1000, RET (rabbit; Immuno-Biological Labs, Japan) 1:50, GFAP (rabbit; DAKO, USA) 1:400, phospho-histone-3 (PH3) (rabbit; Upstate) 1:500, SOX10 (mouse; kindly provided by Dr David Anderson) (Lo et al., 2002) 1:10, Mash1 (mouse; from D. Anderson) (Lo et al., 1991) 1:1, GFP antibody (mouse or rabbit; Molecular Probes) both 1:1000. Cultures were incubated with primary antibodies at RT (for 5-6 hours) or at 4°C (overnight). After several washes with PBT, secondary antibodies were added in blocking solution for 2-4 hours at RT at the following dilutions: anti-mouse FITC-conjugated (Jackson labs) 1:500, anti-rabbit FITC-conjugated (Jackson labs) 1:500, anti-mouse AlexaFlour (Molecular Probes) 1:500, anti rabbit Alexa Flour (Molecular Probes) 1:500. Preparations were counter stained with TO-PRO-3-iodide (Molecular Probes; 1:3000 in PBS) and mounted using Vectashield™ (Vector Laboratories) or mounted directly in Vectashield containing DAPI. Immunostained cultures were examined with a Bio-Rad confocal microscope or a Zeiss epifluorescence microscope (Axiophot). Images were analysed using Metaphor software package (Universal Imaging) and figures were compiled using Adobe Photoshop 7.

## Results

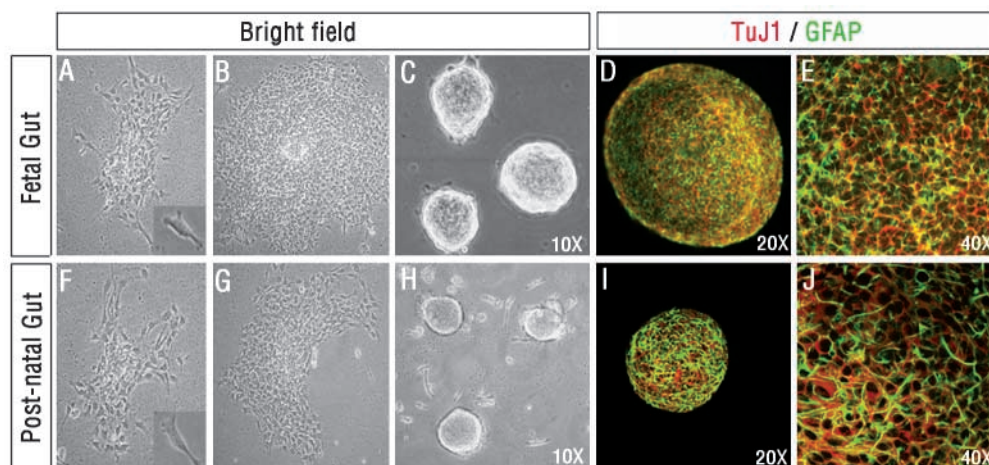
### Neuron and glia-containing neurosphere-like bodies form in primary cultures of dissociated gut

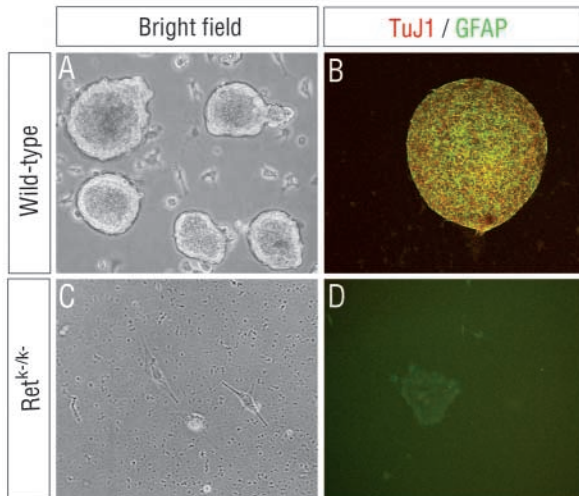
As a first step towards establishing a cell culture protocol that would enrich for ENS progenitors, gut from E11.5 mouse

embryos was dissociated into near single cell suspension and cultured in a medium that supports growth of NCSCs (Morrison et al., 1999; Stemple and Anderson, 1992). Immunostaining of such cultures using neuronal and glial markers [TuJ1 and glial fibrillary acidic protein (GFAP), respectively] on day 1, failed to identify any neurons or glial cells (data not shown). Two to three days later, characteristic colonies appeared that were composed mostly of flat cells with short cytoplasmic processes (Fig. 1A). By day 7, these colonies were considerably larger and their cells started to pile up (Fig. 1B) and by day 10 formed spherical structures which eventually detached and floated in the medium (Fig. 1C). These structures, which we termed neurosphere-like bodies (NLBs), continued to grow in size and contained large numbers of neurons and glia (Fig. 1D,E). This, together with the absence of TuJ1+ and GFAP+ cells during the immediate postplating period, suggested that the majority of neurons and glia of NLBs were generated in vitro from undifferentiated progenitors.

Next we examined whether similar NLBs formed in cultures of postnatal gut. For this, the outer smooth muscle layers along with the myenteric plexus were dissected from the small intestine of 2- to 14-day-old mice, dissociated and plated under conditions identical to those used for foetal gut cultures. Characteristic NCSC-like colonies and NLBs also formed in cultures derived from all postnatal stages we tested with a time course similar to that observed in foetal cultures (Fig. 1F-H). However, the size of postnatal NLBs was generally smaller relative to the foetal ones (Fig. 1, compare H and I with C and D, respectively). Immunostaining of NLBs from day-10 postnatal cultures identified large numbers of neurons and glia (Fig. 1I,J). This, together with the absence of both cell types in the immediate postplating period (24 hours), suggested that our culture conditions did not favour the attachment or growth of intrinsic gut neurons and glia but supported the in vitro differentiation of neurogenic and gliogenic progenitors. Staining with antibodies against alpha smooth muscle actin (SMA) (Owens, 1998) showed that a small fraction of cells in both foetal and postnatal NLBs (1-5%) were smooth muscle cells. Also, a fraction of NLB cells (5-15%) were negative for all differentiation markers used, raising the possibility that they represent undifferentiated progenitors. Finally, neuron- and

**Fig. 1.** Formation of NLBs in cultures of dissociated foetal and postnatal gut. Whole gut from E11.5 mouse embryos (A-E) or the outer muscle layers from P2-14 gut (F-J) were dissociated and plated in NCSC medium. 2-3 days later characteristic colonies appeared in both foetal and postnatal gut cultures (A and F, respectively) which were composed of flat cells with small processes. Higher magnification of individual cells is shown in the insets. Over the next 3-4 days colonies grew in size (B,G) and eventually formed NLBs which detached and floated in the medium by day 10 (C,H). Immunostaining with antibodies against TuJ1 and GFAP indicated that NLBs from both foetal (D,E) and postnatal (I,J) cultures contained large numbers of neurons and glia. E and J are enlargements of parts of the NLBs shown in D and I, respectively. Note that postnatal NLBs were reproducibly smaller relative to their foetal counterparts.





**Fig. 2.** NLBs do not form in gut cultures from *Ret<sup>k-</sup>* homozygous embryos. Cultures were established from individual guts dissected from E11.5 embryos from *Ret<sup>+/-</sup>* heterozygous parents. Neuron- and glia-containing NLBs formed readily in cultures of wild-type (A,B) and *Ret<sup>+/-</sup>* (not shown) embryos but not in cultures from *Ret<sup>k-</sup>* homozygotes (C,D).

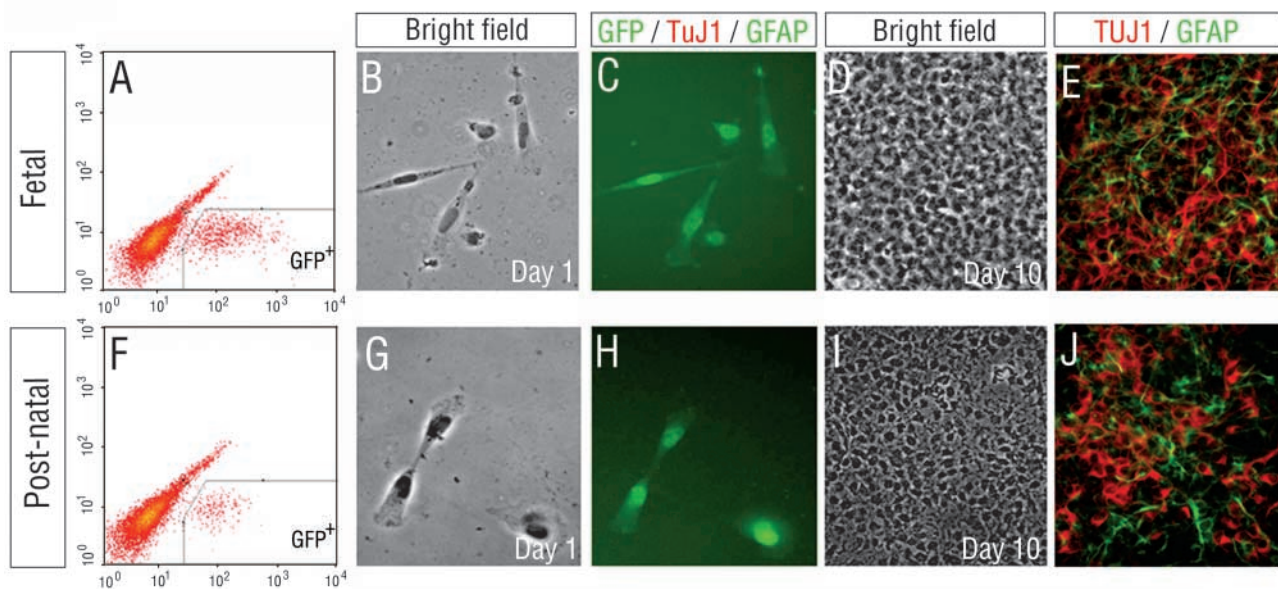
glia-containing secondary and tertiary NLBs formed upon dissociation and replating of a small fraction of cells from primary foetal and postnatal NLBs (data not shown).

During embryogenesis, all enteric neurons and glia originate from neural crest-derived progenitors (Le Douarin, 1999; Yntema and Hammond, 1954). To confirm that NLBs were also derived from cells of neural crest origin, we examined gut cultures from RET-deficient animals in which the

gastrointestinal tract fails to be colonised by ENCCs (Durbec et al., 1996; Schuchardt et al., 1994). Intestine was dissected from individual progeny of intercrossed *Ret<sup>k-</sup>* heterozygotes and cultured as described above. As expected, intestine from wild-type (*Ret<sup>+/+</sup>*) or heterozygous (*Ret<sup>+/-</sup>*) embryos or neonates formed NLBs with many TuJ1+ and GFAP+ cells (Fig. 2A,B and data not shown). In contrast, neither NCSC-like colonies nor NLBs were present in gut cultures generated from *Ret<sup>k-k-</sup>* animals (Fig. 2C). Furthermore, staining of mutant cultures for TuJ1 and GFAP, failed to identify any neurons or glial cells (Fig. 2D). In summary, our experiments suggest that the culture conditions we have employed here support the expansion, propagation and differentiation of neurogenic and gliogenic progenitors from foetal and postnatal gut.

### Isolation of progenitors of enteric neurons and glia from NLBs

To isolate ENS progenitors from NLBs and study their properties in vitro, we selectively marked these cells by expression of a fluorescent tag. NLBs were dissociated and infected with a green fluorescent protein (GFP)-expressing retrovirus, the genome of which integrates permanently into proliferating cells (Brown, 1997). We reasoned that isolation of GFP-expressing cells shortly after infection would enrich for dividing cells, the subpopulation most likely to include progenitors. Flow cytometric profiles of retrovirally transduced foetal and postnatal NLBs indicated that 24 hours after infection a significant fraction of cells expressed GFP (Fig. 3A,F). The proportion of GFP-expressing cells in foetal NLBs ( $8.3 \pm 4.9\%$ ) was reproducibly higher relative to that of their postnatal counterparts ( $4.2 \pm 2.6\%$ ), suggesting that they contain a higher percentage of dividing cells. Immunostaining of the flow cytometrically isolated cells shortly after replating



**Fig. 3.** Retrovirally transduced NLBs yield undifferentiated cells which proliferate and differentiate into neurons and glia. (A,F) 24 hours after infection, GFP-expressing cells were isolated by fluorescence activated cell sorting and replated. (B,G) Bright-field images of GFP+ cells 12 hours after plating from foetal and postnatal NLBs, respectively. (C,H) Fluorescent images of the same cells immunostained for TuJ1 and GFAP. Note that the only fluorescent signal detected is that of nuclear localised GFP. (D,I) Bright-field images of GFP+ cells isolated from foetal and postnatal NLBs and cultured for 10 days. (E,J) The corresponding fluorescent images after immunostaining for TuJ1 and GFAP. Note the presence of large numbers of neurons and glia at this stage.

revealed that they lack mature neuronal or glial markers (Fig. 3B,C,G,H). However, these cells were able to proliferate and differentiate into neurons and glia (Fig. 3D,E,I,J).

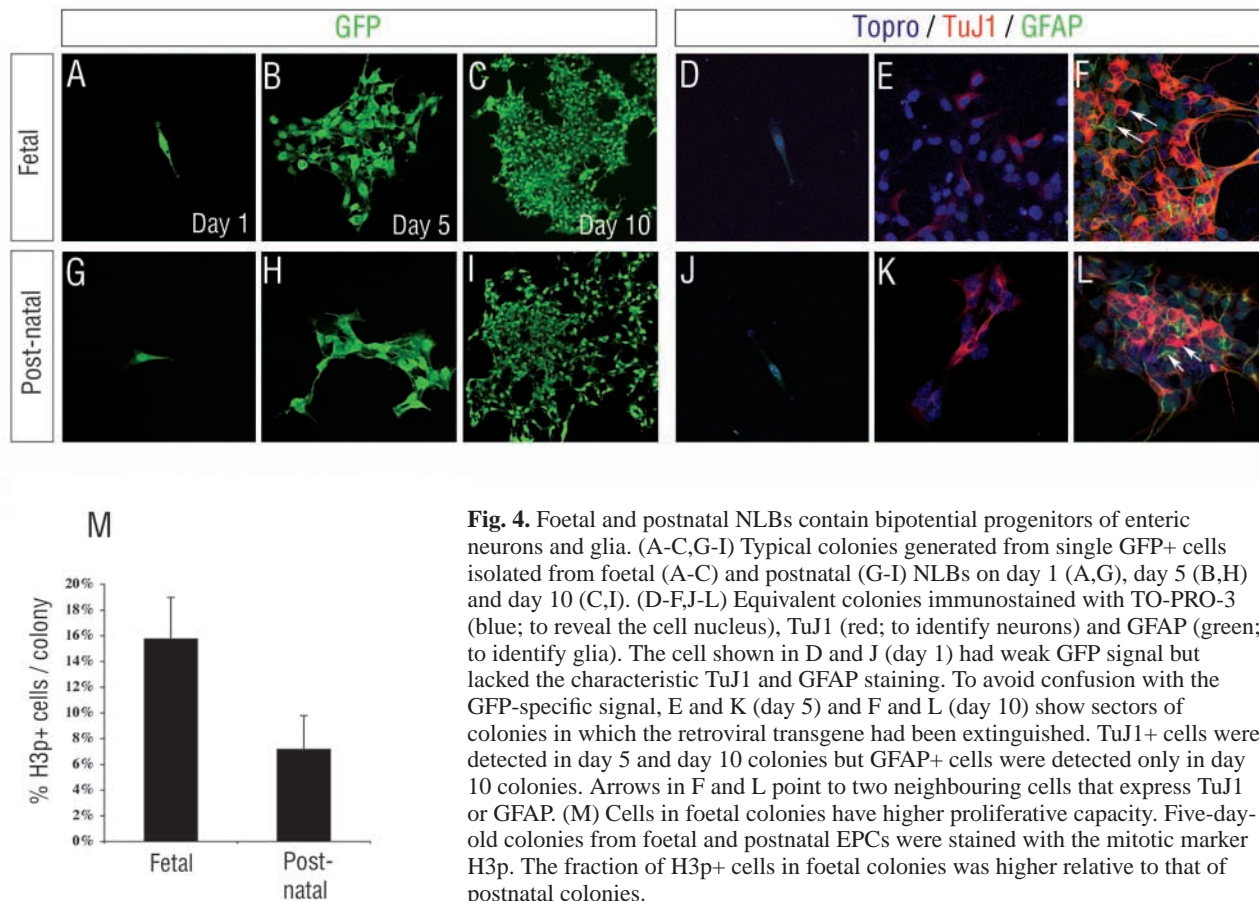
To examine whether the flow cytometrically isolated cells contain multipotential progenitors, GFP+ cells were cultured at clonal density for up to 15 days. Phenotypic analysis of the resulting colonies at the end of the culture period indicated the developmental potential of the founder cells. Approximately 25% of GFP+ cells isolated from foetal NLBs were capable of generating colonies, the size of which increased throughout the culture period (Fig. 4A-C). The rest of the cells remained single or died. Freshly isolated (day 1) GFP+ cells did not express TuJ1 or GFAP (Fig. 4D). However, 4 days later (day 5;  $64 \pm 18$  cells/colony), 82% of colonies contained a small number of TuJ1+ cells (approximately 10% of the cells) but no colonies had GFAP+ cells (Fig. 4E). On day 10 (>300 cells/colony), all colonies contained a relatively large number of TuJ1+ cells (approx. 25% of cells) while the majority (87%) of colonies also contained mature glial cells (Fig. 4F). No overlap between neuron-specific tubulin and GFAP-expressing cells was observed (Fig. 4; arrows in F point to two neighbouring cells expressing GFAP and TuJ1, respectively).

GFP+ cells isolated from postnatal NLBs were also capable of forming colonies with similar clonogenic efficiency (25%; Fig. 4G,I). In addition, the time course of neuronal and glial differentiation appeared to be similar to that of the foetal cells (Fig. 4G-I). No neurons or glia were present in cultures on day 1 (Fig. 4J) but by day 5 ( $44 \pm 10$  cells/colony), TuJ1+ cells were

present in the majority of colonies (Fig. 4K). At this stage we did not detect any GFAP-expressing cells (Fig. 4K). By day 10 (>100 cells/colony), 89% of the colonies contained both TuJ1+ and GFAP+ cells (Fig. 4L). Generally, GFP+ cells isolated from postnatal NLBs generated smaller colonies, consistent with a relatively reduced proliferative capacity of these cells. To directly test the mitotic activity of the two clonogenic cell populations, colonies were fixed 5 days after plating and immunostained for the mitotic marker phosphohistone-3 (H3p) (Mahadevan et al., 1991). We observed that the percentage of H3p+ cells within foetal and postnatal colonies was 15.8% and 7.2%, respectively (Fig. 4M). In summary, these experiments indicated that the flow cytometrically isolated GFP+ cells from foetal and postnatal NLBs include a large fraction of bipotential progenitors of enteric neurons and glia. We termed these progenitors, ENS progenitor cells (EPCs).

### EPCs recapitulate the ontogenetic profile of ENS progenitors

To further characterise the EPC colonies, GFP+ cells were isolated from NLBs, plated at clonal densities and analysed 1, 3 and 10 days after plating for expression of several molecular markers that define various stages of cellular differentiation in the ENS. On day 1, approximately 25% of foetal EPCs expressed *Sox10* (Fig. 5A1-A2). This percentage matches the clonogenic efficiency of these cells and suggests that only *Sox10*-expressing GFP+ cells have the potential to form multilineage colonies. Immunostaining for MASH1 and RET identified no positive cells at this stage (Fig. 5B1-C2) which,



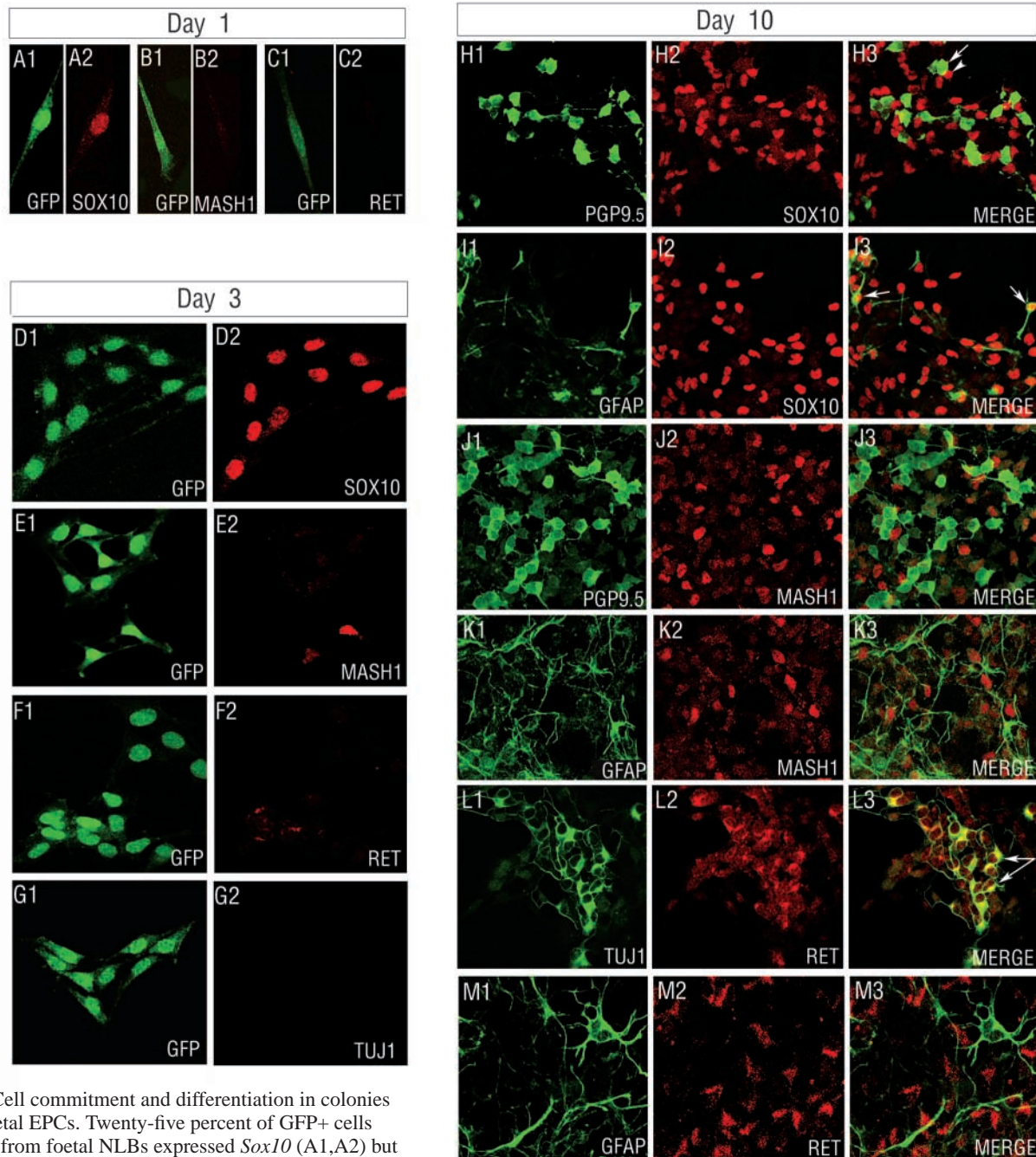
**Fig. 4.** Foetal and postnatal NLBs contain bipotential progenitors of enteric neurons and glia. (A-C,G-I) Typical colonies generated from single GFP+ cells isolated from foetal (A-C) and postnatal (G-I) NLBs on day 1 (A,G), day 5 (B,H) and day 10 (C,I). (D-F,J-L) Equivalent colonies immunostained with TO-PRO-3 (blue; to reveal the cell nucleus), TuJ1 (red; to identify neurons) and GFAP (green; to identify glia). The cell shown in D and J (day 1) had weak GFP signal but lacked the characteristic TuJ1 and GFAP staining. To avoid confusion with the GFP-specific signal, E and K (day 5) and F and L (day 10) show sectors of colonies in which the retroviral transgene had been extinguished. TuJ1+ cells were detected in day 5 and day 10 colonies but GFAP+ cells were detected only in day 10 colonies. Arrows in F and L point to two neighbouring cells that express TuJ1 or GFAP. (M) Cells in foetal colonies have higher proliferative capacity. Five-day-old colonies from foetal and postnatal EPCs were stained with the mitotic marker H3p. The fraction of H3p+ cells in foetal colonies was higher relative to that of postnatal colonies.

together with the virtual absence of TuJ1 and GFAP staining (Fig. 4D,J) suggested that, when isolated from NLBs, EPCs expressed only markers of undifferentiated progenitors but lacked markers of neuronal commitment and neuronal or glial differentiation.

On day 3, only colony-associated cells were positive for SOX10 (Fig. 5D1,D2), further supporting the idea that *Sox10*

expression identified clonogenic cells. At this stage, *Mash1* and *Ret* were expressed by a small subset of cells in 74% of colonies (Fig. 5E1-F2) but we detected no TuJ1+ or GFAP+ cells (Fig. 5G1,G2 and data not shown).

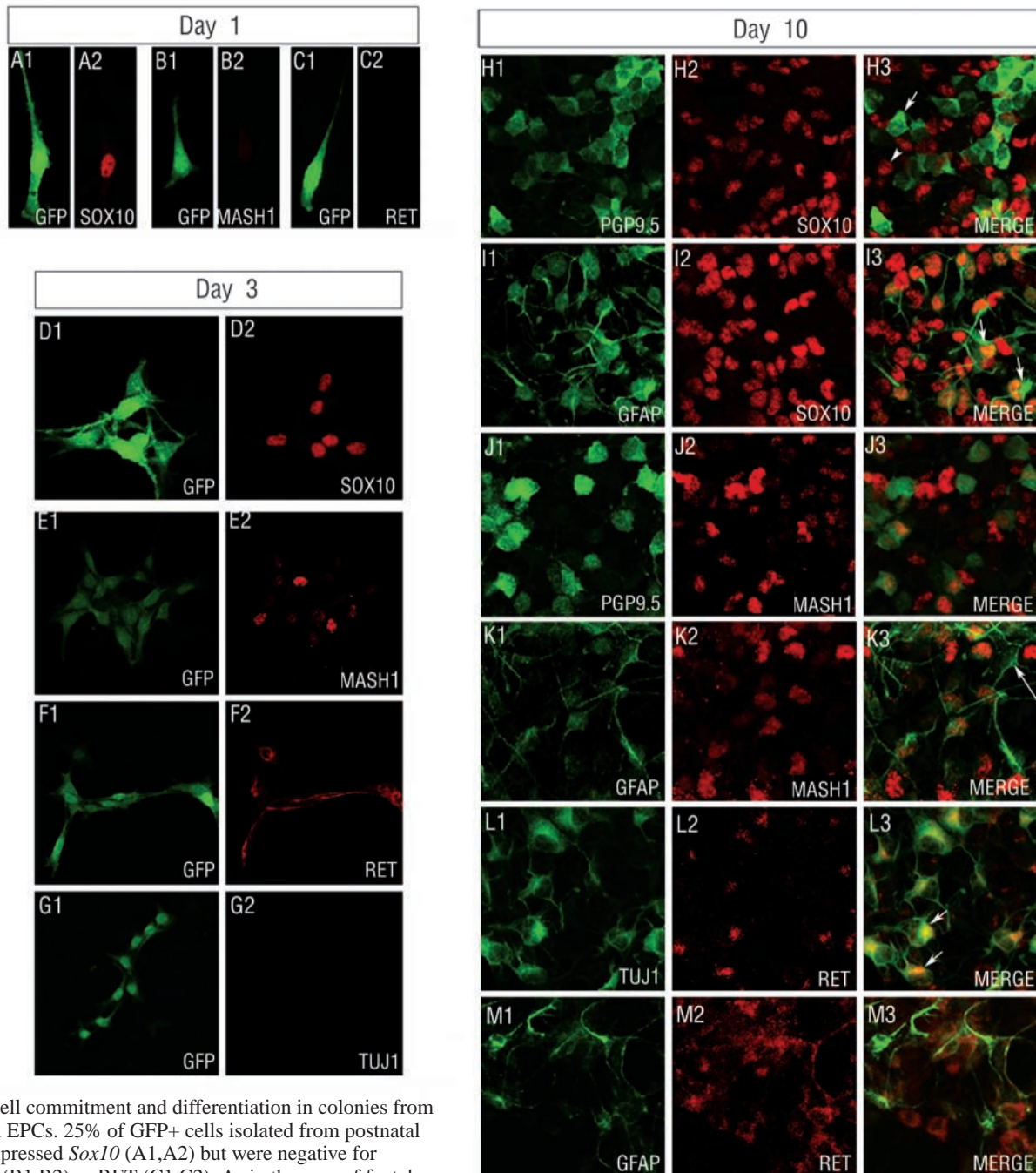
On day 10, 25% of cells in all colonies were positive for the neuronal markers TuJ1 (Fig. 4) and PGP9.5 (Fig. 5H1). Double labelling experiments showed that all PGP9.5-expressing cells



**Fig. 5.** Cell commitment and differentiation in colonies from foetal EPCs. Twenty-five percent of GFP+ cells isolated from foetal NLBs expressed *Sox10* (A1,A2) but were negative for MASH1 (B1,B2) or RET (C1,C2). On day 3, *Sox10* expression was detected only in colony-associated cells (D1,D2), suggesting strongly that only the GFP+/SOX10+ EPCs have clonogenic potential. At this stage, only a subset of cells expressed *Mash1* (E1,E2) or *Ret* (F1,F2) but we detected no TuJ1+ cells (G1,G2). On day 10, *Sox10* was expressed in a subset of cells in the colonies (H2,I2). At this stage, all colonies contained PGP9.5+ cells (H1) which were negative for SOX10 (H3; arrow points to a PGP9.5+/SOX10- cell and arrowhead points to a neighbouring PGP9.5-/SOX10+ cell). Also, at this stage 87% of the colonies contained GFAP+ cells (I1) which maintained expression of *Sox10* (I3; arrows point to GFAP+/SOX10+ cells). Cells expressing high levels of PGP9.5 were negative for MASH1 (J1-J3). Also, GFAP and MASH1 are present on mutually exclusive cells (K1-K3). Finally, the highest levels of RET were detected in TuJ1+ cells (L1-L3; arrows in L3) but GFAP+ cells did not express *Ret* (M1-M3).

were negative for SOX10 (Fig. 5H1-H3). Also, cells expressing relatively low levels of PGP9.5 (PGP9.5<sup>low</sup>) co-expressed *Mash1* but the vast majority of PGP9.5<sup>high</sup> cells were negative for MASH1 (Fig. 5J1-J3). Finally, cells expressing TuJ1, also expressed the highest levels of *Ret* (Fig. 5L1-L3). In addition to neuronal markers, 87% of day-10 foetal EPC colonies

included cells expressing GFAP. Double staining showed that GFAP<sup>+</sup> cells were generally SOX10<sup>+</sup>, but a large fraction of SOX10<sup>+</sup> cells were negative for GFAP (Fig. 5I1-I3). As no mature neurons expressed SOX10, these data are consistent with the idea that this transcriptional regulator is expressed in undifferentiated EPCs and their gliogenic progeny. Also,



**Fig. 6.** Cell commitment and differentiation in colonies from postnatal EPCs. 25% of GFP<sup>+</sup> cells isolated from postnatal NLBs expressed *Sox10* (A1,A2) but were negative for MASH1 (B1,B2) or RET (C1,C2). As in the case of foetal EPCs, on day 3 *Sox10* expression was detected only in colony-associated cells (D1,D2). At this stage, a subset of cells expressed *Mash1* (E1,E2) or *Ret* (F1,F2) but we detected no TuJ1<sup>+</sup> cells (G1,G2). On day 10, *Sox10* was expressed in a subset of cells (H2,I2) and all colonies contained PGP9.5<sup>+</sup> cells (H1) which were negative for SOX10 (H3; arrow points to a PGP9.5<sup>+</sup>/SOX10<sup>-</sup> cell and arrowhead points to a PGP9.5<sup>-</sup>/SOX10<sup>+</sup> cell). Also, at this stage, 89% of the colonies contained GFAP<sup>+</sup> cells (I1) which maintained expression of *Sox10* (I3; arrows point to GFAP<sup>+</sup>/SOX10<sup>+</sup> cells). Cells expressing high levels of PGP9.5 were negative for MASH1 (J1-J3). Also, GFAP and MASH1 were present on mutually exclusive cells (K1-K3; arrow points to a GFAP<sup>+</sup>/MASH1<sup>-</sup> cell). Finally, as for foetal EPC colonies, the highest levels of RET were detected in TuJ1<sup>+</sup> cells (L1-L3; arrows in L3) while *Ret* was not expressed in GFAP<sup>+</sup> cells (M1-M3).

GFAP was absent from cells expressing high levels of *Mash1* or *Ret* (Fig. 5K1-K3 and M1-M3).

The molecular mechanisms underlying differentiation of postnatal EPCs were likely to be similar to those operating in their foetal counterparts. Thus, on day 1, 25% of GFP+ cells isolated from postnatal NLBs were expressing *Sox10* but all cells at this stage were negative for MASH1 and RET (Fig. 6A1-C2). On day 3, all colony-associated cells maintained expression of *Sox10*, a subset of them induced *Mash1* and *Ret* but no TuJ1 was detected (Fig. 6D1-G2). By day 10, a large number of PGP9.5+/SOX10- neurons were present in colonies (Fig. 6H1-H3), most of which also contained GFAP+/SOX10+ cells (Fig. 6I1-I3). Also, the relative expression of PGP9.5, TuJ1, MASH1, RET and GFAP at this stage was similar to that of foetal EPC colonies (Fig. 6J1-M3). Taken together, our findings indicate that clonogenic EPCs isolated from foetal and postnatal NLBs probably represent undifferentiated bipotential ENS progenitors. As is the case for the in vivo progenitors, cultured EPCs initially acquire markers of neurogenic and gliogenic commitment and eventually differentiate into mature neurons and glia.

### EPCs differentiate into a variety of ENS-appropriate neuronal subtypes

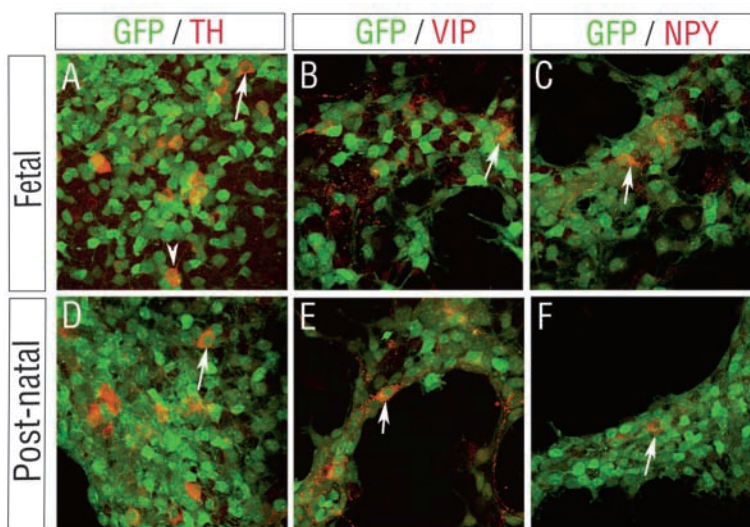
We next examined whether EPCs can generate neuronal subtypes that are normally encountered in the mammalian ENS. For this, clonogenic cultures of EPCs were analysed by immunostaining for a series of molecular markers expressed by overlapping subsets of ENS progenitors and neurons. Among these markers were tyrosine hydroxylase (TH), vasoactive intestinal peptide (VIP), neuropeptide-Y (NPY) and calcitonin gene-related peptide (CGRP). TH, the rate-limiting enzyme in the biosynthesis of catecholamines, is a characteristic marker of adrenergic neurons and is expressed

transiently by a large proportion of vagal neural crest-derived cells migrating in the gut of rodent embryos (transient catecholaminergic cells) (Baetge and Gershon, 1989; Baetge et al., 1990). VIP, NPY and CGRP are neuropeptides that are expressed by specific subpopulations of enteric neurons in the mammalian ENS (Gershon et al., 1994). We found that TH was expressed by a subset of cells in the majority of foetal EPC colonies (Fig. 7A). Most of the positive cells had clear neuronal morphology (arrowhead in Fig. 7A) and co-expressed TuJ1 (not shown) indicating that at least a subset of TH-expressing cells represent mature neurons. Surprisingly, and despite the lack of TH expression in the ENS of rodents after birth, TH+ cells were also detected in a similar percentage of colonies from postnatal EPCs (Fig. 7D). In addition to TH, colonies from both foetal and postnatal EPCs also had VIP-, NPY- and CGRP-expressing cells (Fig. 7B,C,E,F and data not shown). These findings show that in addition to the main restriction along the neuronal and glial cell lineages, EPCs can differentiate into neuronal subtypes encountered in the mammalian ENS.

### Response of EPCs and their progeny to GDNF

GDNF provides the main proliferative and differentiation signals for ENCCs but it is also important for the survival and migration of ENS progenitors (Gianino et al., 2003; Natarajan et al., 2002; Taraviras et al., 1999; Young et al., 2001). To examine the effect of this signalling molecule on the proliferation of EPCs and their progeny, clonogenic cultures of GFP+ cells isolated from foetal and postnatal NLBs were established in standard medium in the presence or absence of GDNF (10 ng/ml). Cell proliferation in the resulting colonies was analysed 5 days later using the mitotic marker H3p. We found that in the presence of GDNF, colonies from both foetal and postnatal EPCs had a significantly higher fraction of H3p+ cells relative to colonies maintained in control medium (Fig. 8, upper part of A). Consistent with this, we observed an increase in the number of cells per colony. Thus, on day 5, the number of cells present in foetal and postnatal EPC colonies maintained in control medium was  $74 \pm 10$  and  $53 \pm 7$  cells, respectively. In the presence of GDNF, the corresponding numbers increased to  $108 \pm 16$  and  $98 \pm 19$ . Concomitant to the increase in cell proliferation, addition of GDNF in complete medium reduced the fraction of TuJ1+ cells (Fig. 8, lower part of A).

We next examined the effect of GDNF on EPC colonies in the absence of growth factors and CEE, a probable source of multiple signalling molecules. For this, clonogenic cultures of EPCs were established in standard medium. Four days later, half of the cultures were switched to medium lacking growth factors and CEE (defined medium) or to defined medium supplemented with 10 ng/ml of GDNF and cultured for a further 3-4 days. Significant neuronal differentiation was observed in both types of cultures. However, in cultures maintained in the presence of GDNF, neurites were dramatically longer and highly branched. In contrast, in cultures maintained in defined medium, neurons had relatively short and non-branched axons (Fig. 8B-E). Taken together, these



**Fig. 7.** EPCs differentiate into ENS-appropriate neuronal subtypes. 15-day-old colonies established from foetal (A-C) and postnatal (D-F) EPCs were fixed and immunostained with antibodies for GFP (green; all panels) and TH (red; A,D), VIP (red; B,E) and NPY (red; C,F). Subsets of neurons expressing these markers (arrows in all panels) were generated with similar efficiencies in both foetal and postnatal colonies. Arrowhead in A points to a neurite-bearing TH+ cell.



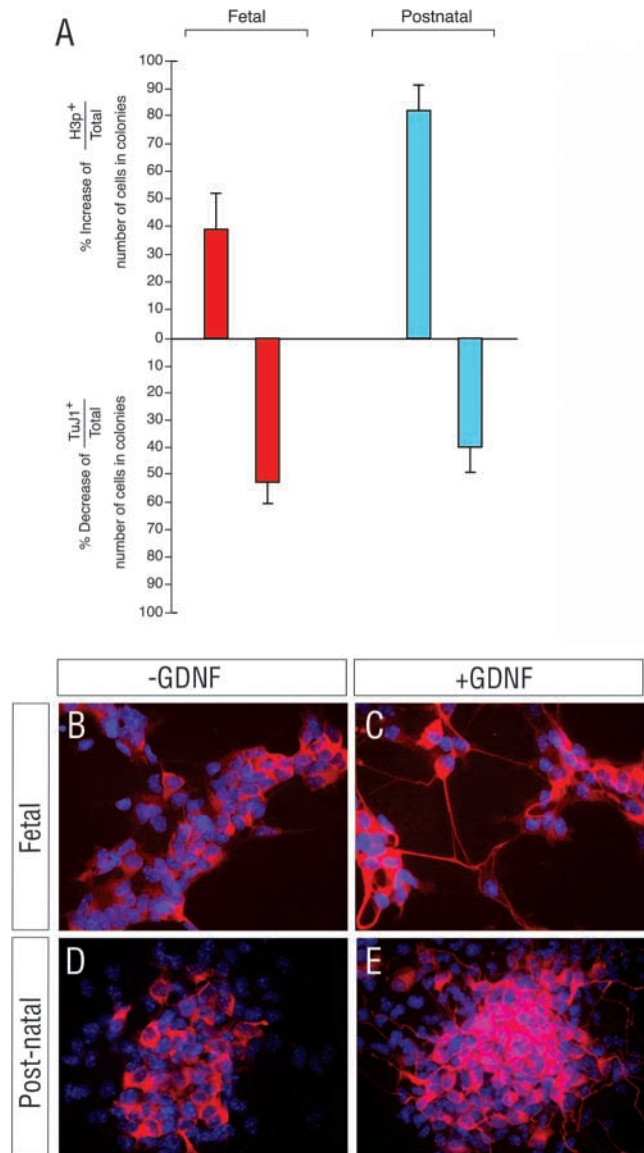
studies indicate that GDNF promotes the morphological differentiation of foetal and postnatal EPC progeny in a manner similar to that of ENS progenitors (Taraviras et al., 1999; Taraviras and Pachnis, 1999).

### EPCs differentiate into enteric neurons and glia upon grafting into foetal gut in organ culture

We next tested the ability of NLB cells to colonise foetal gut. For this, manually cut pieces of foetal or postnatal NLBs were labelled with DiI and grafted into the wall of guts dissected from E11.5 wild-type mouse embryos and cultured organotypically for 4 days, as described previously (Natarajan et al., 1999). Emigration of cells from both foetal or postnatal NLBs was initiated within 24 hours of grafting and continued until an extensive halo of DiI-positive cells formed around the site of engraftment (data not shown). Extensive migration of DiI-labelled cells was also observed from foetal or postnatal NLBs grafted into guts isolated from *Ret<sup>k-</sup>* mutant embryos, which fail to develop intrinsic enteric neurons and glia. In addition, immunostaining of grafted mutant guts for TuJ1 or GFAP revealed the presence of large numbers of neurons and glia in the domains that had been colonised by DiI-labelled cells (Fig. 9B and data not shown). As expected, such differentiated cells were absent from non-grafted mutant guts (Fig. 9A). These experiments indicated that neuronal and glial cells originating in foetal and postnatal NLBs can colonise wild-type and aganglionic gut in organ culture.

The previous experiments did not allow us to determine whether the NLB-derived neurons and glia detected in the gut were generated from undifferentiated progenitors or pre-existed within the grafted NLBs. To test the ability of EPCs to generate neurons and glia upon transplantation into the gut wall, GFP+ cells (20-30) isolated from foetal NLBs were grafted into the stomach or intestinal wall of guts isolated from E11.5 wild-type embryos and maintained in organotypic culture. After 14 days in culture, guts were fixed, sectioned and processed for double immunostaining with anti-GFP antibodies (to identify NLB-derived cells) and TuJ1 or GFAP (to identify neurons and glia, respectively). As expected, no GFP+ cells were present in uninjected guts (Fig. 9C). However, GFP-expressing cells were detected in the majority of grafted guts (79%,  $n=34$ ; Fig. 9D). In most cases, single or small groups of GFP+ cells were detected at a distance from the site of injection. Furthermore, the emigrating GFP+ cells were often found in the appropriate location, namely within the smooth muscle cell layers of the outer gut wall (Fig. 9D). 88% of the guts that were successfully transplanted ( $n=27$ ) with EPCs, also contained several GFP+/TuJ1+ cells (on average 2-4 cells per section), while in 74% of these guts, we also identified GFP+/GFAP+ cells (Fig. 9E,F). These data indicate that foetal EPCs are capable of differentiating into neurons and glia upon transplantation into foetal gut in culture.

To examine the ability of postnatal EPCs to integrate into the intrinsic ENS and differentiate into mature neurons and glia, similar transplantation experiments were carried out using GFP+ cells isolated from postnatal NLBs. These experiments established that, similarly to foetal EPCs, postnatal EPCs were capable of colonising the gut (67%,  $n=34$ ) and migrating along the anteroposterior and radial axis of the gut wall (Fig. 9J). In



**Fig. 8.** Effects of GDNF on the progeny of foetal and postnatal EPCs. (A) Day-5 clonal cultures of EPCs fixed and stained for H3p or TuJ1. In the upper part of the graph, bars represent the percentage increase in the number of H3p+ cells in the presence of GDNF relative to those present in control (absence of GDNF) culture conditions. In the lower part of the graph, bars represent the percentage decrease in the number of TuJ1+ cells in the presence of GDNF relative to those present in control cultures. (B-E) GDNF also promotes the morphological differentiation of EPC progeny maintained in defined medium. Foetal (B,C) and postnatal (D,E) EPCs were cultured for 4 days in complete NCSC medium. Subsequently, they were cultured for a further 4 days in defined medium (NCSC medium lacking CEE, FGF and EGF) in the absence (B,D) or presence (C,E) of GDNF. GDNF promotes axonal growth and branching of neurons in both foetal and postnatal EPC colonies.

addition, in 73% and 61% of the successfully transplanted guts, we observed GFP+ cells differentiating into neurons (GFP+/TuJ1+) and glia (GFP+/GFAP+), respectively (Fig. 9K,L).

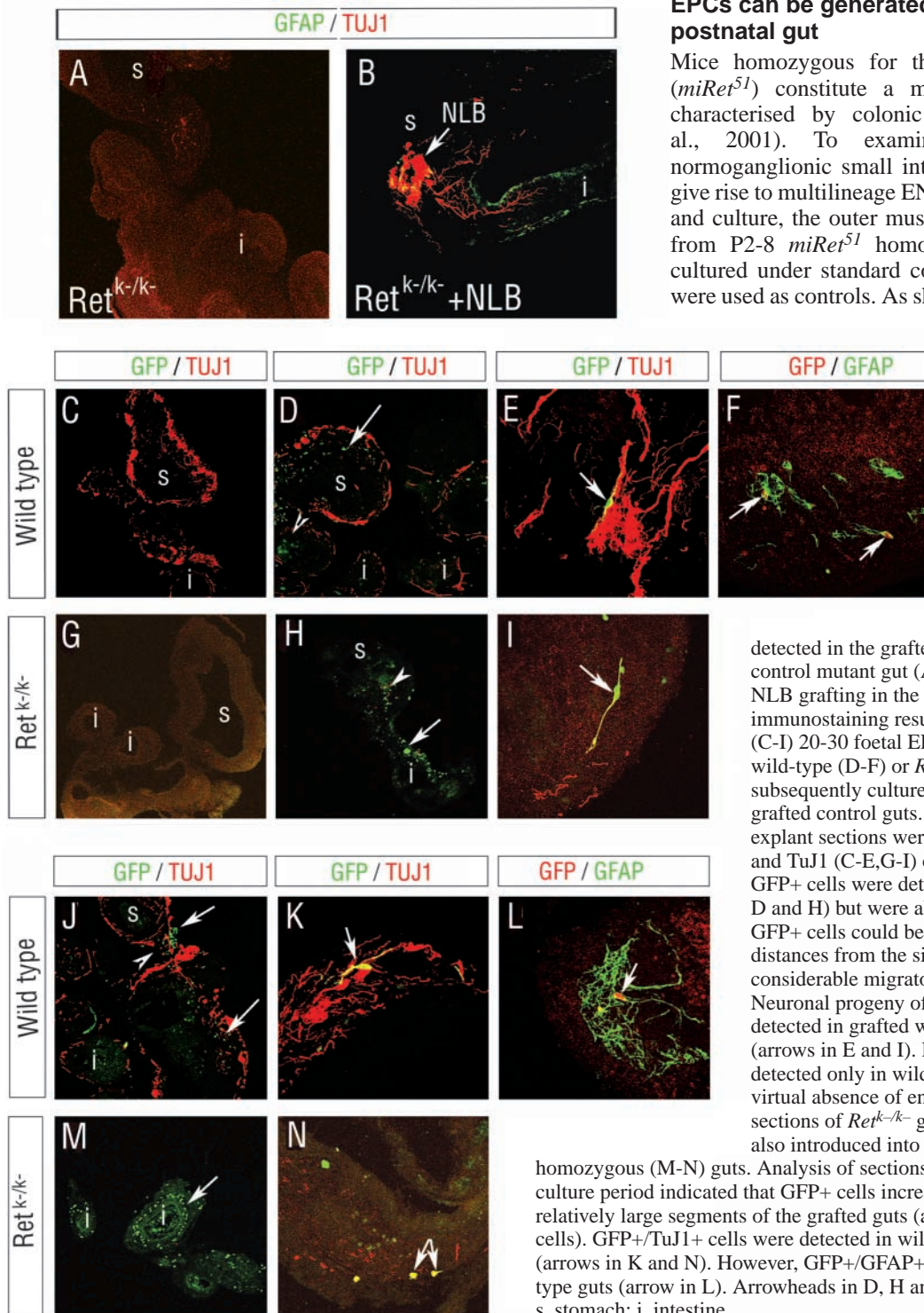
We also tested the ability of EPCs to colonise aganglionic

gut in organotypic culture. For this, the gut of *Ret<sup>k-/k-</sup>* embryos was transplanted with foetal EPCs or postnatal EPCs and analysed by double immunostaining as above. We found that similar to the transplantations into wild-type gut, both the foetal and postnatal EPCs were capable of recolonising aganglionic gut mesenchyme with an efficiency comparable to those observed for wild-type gut (83%  $n=6$  and 60%  $n=5$ , respectively; Fig. 9H,M). Furthermore, both foetal and postnatal EPCs were capable of differentiating into neurons

(Fig. 9I,N). However, we were unable to detect double positive GFP+/GFAP+ cells (not shown). In summary, our experiments show that both foetal and postnatal EPCs have the potential to colonise the wall of wild-type and aganglionic gut. In addition, both types of progenitors can generate neuronal and glial progeny upon transplantation into wild-type gut wall. However, under our present experimental conditions, grafting into aganglionic gut resulted in the generation of neurons only.

### EPCs can be generated from partially aganglionic postnatal gut

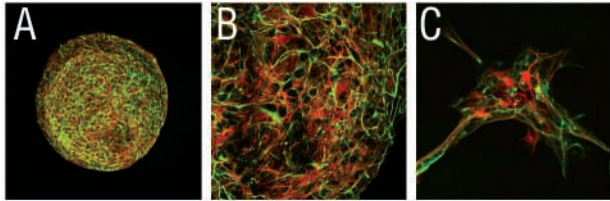
Mice homozygous for the hypomorphic allele of *Ret* (*miRet<sup>51</sup>*) constitute a model for HSCR as they are characterised by colonic aganglionosis (de Graaff et al., 2001). To examine whether the apparently normoganglionic small intestine of *miRet<sup>51</sup>* neonates can give rise to multilineage ENS progenitors upon dissociation and culture, the outer muscle layers of the small intestine from P2-8 *miRet<sup>51</sup>* homozygotes were dissociated and cultured under standard conditions. Wild-type littermates were used as controls. As shown in Fig. 10A,B, gut cultures



**Fig. 9.** EPCs colonise foetal gut and have the potential to generate neurons and glia. (A,B) DiI-labelled pieces of foetal and postnatal NLBs were grafted into the wall of gut dissected from *Ret<sup>k-/k-</sup>* E11.5 mouse embryos and maintained in organotypic culture. A large number of neurons and glial cells were

detected in the grafted gut (B) but were absent from control mutant gut (A). Arrow in B points to the site of NLB grafting in the stomach (s). Processing of guts for immunostaining resulted in loss of DiI fluorescence. (C-I) 20-30 foetal EPCs were grafted into the wall of wild-type (D-F) or *Ret<sup>k-/k-</sup>* (H-I) guts which were subsequently cultured for 14 days. C and G are non-grafted control guts. At the end of the culture period, explant sections were immunostained for GFP (C-I) and TuJ1 (C-E,G-I) or GFAP (F). Large numbers of GFP+ cells were detected in the grafted guts (arrows in D and H) but were absent from control guts (C,G). GFP+ cells could be detected at relatively large distances from the site of injection suggesting a considerable migratory ability of grafted cells. Neuronal progeny of EPCs (GFP+/TuJ1+ cells) were detected in grafted wild-type and RET-deficient guts (arrows in E and I). However, GFP+/GFAP+ cells were detected only in wild-type guts (arrows in F). Note the virtual absence of endogenous TuJ1 signal from sections of *Ret<sup>k-/k-</sup>* guts (G). (J-N) Postnatal EPCs were also introduced into wild-type (J-L) and *Ret<sup>k-</sup>*

homozygous (M-N) guts. Analysis of sections from grafted guts at the end of the culture period indicated that GFP+ cells increased in numbers and colonised relatively large segments of the grafted guts (arrows in J and M point to GFP+ cells). GFP+/TuJ1+ cells were detected in wild-type and RET-deficient gut (arrows in K and N). However, GFP+/GFAP+ cells were only detected in wild-type guts (arrow in L). Arrowheads in D, H and J show the site of EPC grafting. s, stomach; i, intestine.



**Fig. 10.** Formation of NLBs from *miRet<sup>51</sup>* homozygous mice. Postnatal gut was isolated from individual P2-8 mice born to *miRet<sup>51</sup>* heterozygous parents, dissociated and cultured as described. (A,B) NLBs containing large numbers of TuJ1+ and GFAP+ cells formed in gut cultures from *miRet<sup>51</sup>* animals. (C) In addition, retroviral transduction of NLBs resulted in the efficient isolation of EPCs which generated neuron- and glia-containing colonies (a sector of which is shown here).

from *miRet<sup>51</sup>* homozygous mutants generated large numbers of NLBs with a time course similar to that of control cultures. In addition, GFP-retrovirus transduction of *miRet<sup>51</sup>* NLBs resulted in the efficient isolation of GFP-expressing cells which in clonogenic assays were capable of generating neurons and glia (Fig. 10C). These data suggest that the normoganglionic intestine of *miRet<sup>51</sup>* homozygous animals is capable of generating EPCs with an efficiency similar to that of wild-type postnatal gut.

## Discussion

### Properties of enteric nervous system progenitors isolated from NLBs

Using cultures of dissociated gut and retrovirus-mediated gene transfer, we have isolated mammalian ENS progenitors (EPCs) capable of generating neurons and glia in vitro and upon grafting into mouse foetal gut maintained in organ culture. EPCs were isolated from both foetal gut and gut from animals up to 2 weeks of age. With the exception of reduced proliferation rate of cells in postnatal EPC colonies relative to their foetal counterparts, the properties and the developmental potential of the two groups of progenitors were similar.

Our strategy for the isolation of EPCs was not dependent on expression of cell surface or intracellular markers on ENS progenitors; instead, it was based exclusively on the ability of neural crest-derived cells in the gut to maintain their proliferative capacity and differentiation potential, leading to formation of NLBs. However, gene expression analysis of EPCs and their progeny in clonogenic assays showed that they undergo changes in patterns of gene expression which reproduce those observed in endogenous ENS progenitors (Young et al., 2003; Young et al., 1999). Thus, similarly to the early migratory vagal neural crest cells, EPCs express *Sox10* but lack detectable levels of RET or lineage specification markers, such as MASH1 (SOX10+/RET-/MASH1-). However, shortly after plating, a subset of EPC progeny express RET and MASH1 and differentiate into mature neurons. SOX10+/RET- progenitor cells had not been identified previously in mouse embryonic or postnatal gut. In a recent study, Young and colleagues showed that the majority of ENS progenitors express *Sox10* and *Ret* at all embryonic stages examined [(Young et al., 2003; Young et al., 1999) and personal communication]. This raises the question of the

cellular origin of EPCs in the cell culture system described here. One possibility is that our protocol preferentially expands a relatively small subpopulation of pre-existing SOX10+/RET-/MASH1- cells which represent early migrating vagal neural crest cells that persist in the mouse gut throughout enteric neurogenesis. An alternative hypothesis would be that our culture conditions reprogram a relatively late ENS progenitor to acquire properties characteristic of an earlier precursor cell type. Such cell reprogramming has been observed in vitro before. For example, under certain culture conditions, oligodendrocyte precursor cells (OPCs) revert to self-renewable multipotential neural stem cells, which can generate neurons, astrocytes and oligodendrocytes (Kondo and Raff, 2000). It is therefore possible that signals in our cultures reprogram neural crest-derived cells in mouse gut to acquire characteristics of NCSCs.

A characteristic property of NCSCs isolated from neural tube explants or prospectively identified from peripheral tissues at later stages of embryogenesis, is their ability to self-renew and generate similar multipotential progeny (Morrison et al., 1999; Stemple and Anderson, 1992). The ability of EPCs to generate multiple lineages (in addition to the neuronal and glial ones) is currently unclear. A small percentage of SMA+/GFP+ cells have been isolated from NLBs but the ability of EPCs to generate myofibroblasts [as is the case for other NCSCs (Morrison et al., 1999)] requires further experimentation. The isolation of bipotential progenitors from primary NLBs at relatively late stages of gut cultures and from secondary and tertiary NLBs, suggests the presence of self-renewing progenitors in these structures. Such progenitors could divide asymmetrically to generate committed neurogenic and gliogenic precursors as well as bipotential progenitors isolated as EPCs. The presence of self-renewing progenitors in NLB cultures is further supported by our recent data indicating that dissociation and replating of primary EPC colonies at clonal densities gives rise to neuron- and glia-containing secondary colonies. Although the formation of such secondary colonies was inefficient (due primarily to failure of cells to survive upon replating) we were able to reproducibly generate four to five multipotential secondary colonies per experiment, thus establishing in principle the ability of EPCs to self renew in vitro. These studies, together with recent experiments indicating that EPCs express *Phox2B* (N.B. and V.P., unpublished data), a locus encoding a homeodomain-containing transcription factor necessary for the specification of autonomic and enteric neural crest cells lineages (Pattyn et al., 1997; Pattyn et al., 1999), suggest that EPCs are likely to represent self-renewing progenitors of the sympathoenteric lineage (Durbec et al., 1996).

In addition to reproducing stages of cell differentiation observed for endogenous ENS progenitors, EPCs and their progeny appear to respond to signals that play a critical role in mammalian ENS development. A series of studies have shown that GDNF controls several aspects of mammalian ENS development by activating RET signalling in ENCCs and their progeny (Taraviras and Pachnis, 1999). Among the known effects of RET activation are the proliferation of ENS progenitors, a response likely to determine the number of enteric neurons in the mature ENS (Gianino et al., 2003) and the morphological differentiation of ENCCs (Taraviras et al., 1999). Consistent with these studies, we observed that GDNF

enhanced cell proliferation and reduced the percentage of neurons in colonies of foetal and postnatal EPCs maintained in complete medium. However, GDNF promoted the morphological differentiation of neurons in the absence of CEE and other growth factors. These findings highlight the context-dependent outcome of RET activation (Barlow et al., 2003) and suggest that additional signalling molecules (such as those present in CEE) could modify the effect of GDNF on EPCs. The exact cell type in EPC colonies that responds to GDNF is presently unclear. Our failure to detect *Ret* expression in EPCs shortly after their isolation suggests that the target of GDNF are committed progenitors, such as the RET<sup>+</sup> cells detected after day 3 of culture and their differentiated progeny. This is consistent with the idea that EPCs represent pre-enteric progenitors similar to the early vagal neural crest cells that migrate and proliferate independently of RET activation (Durbec et al., 1996; Taraviras et al., 1999), but are capable of generating progeny similar to the RET-dependent ENCCs.

The suggestion that EPCs represent progenitors of the mammalian ENS is further supported by their ability to generate neuronal subtypes normally encountered in enteric ganglia. Nearly all colonies of EPCs, generated from either foetal or postnatal stages, contained neurons that expressed NPY, VIP or CGRP. The generation of such neuronal subtypes by foetal and postnatal EPCs is consistent with birthdating studies which indicate that neurons expressing a wide range of neurotransmitters and neuropeptides, including those described here, are born between E9.5 and P15 (Pham et al., 1991). Expression of TH, a characteristic marker of adrenergic neurons, is normally restricted to a subset of foetal ENS progenitors and mature neurons between E9.5 and E14.5 and is absent from postnatal gut (Baetge and Gershon, 1989; Baetge et al., 1990). Our findings that colonies generated from foetal EPCs produce TH<sup>+</sup> neurons further argues that these cells have a developmental potential similar to that of the endogenous ENS progenitors. However, similar numbers of TH<sup>+</sup> cells were also detected in colonies from postnatal EPCs. The reasons of this apparent discrepancy are currently unclear. It is possible TH expression reflects the reprogramming of postnatal cells to become similar to foetal progenitors. Alternatively, the generation of TH<sup>+</sup> neurons from postnatal EPCs results from the absence of a signal that is normally present in the late embryonic and postnatal gut, and represses the expression of TH. Experiments are currently in progress to distinguish between these possibilities.

### Differentiation of foetal and postnatal EPCs upon grafting into foetal gut in organ culture

Grafting of foetal and postnatal NLBs into the wall of foetal gut maintained in organ culture resulted in extensive emigration of cells from the graft and invasion of the surrounding tissue. Such emigration took place in both wild-type gut (not shown) and in gut from RET-deficient embryos which lack endogenous neural crest derivatives. Absence of an intrinsic ENS from the latter allowed us to establish that many of the cells that emigrated from NLBs were mature neuronal and glial cells. These findings suggest that NLBs constitute a repository of enteric neurons and glia that can be used to colonise the wall of aganglionic gut. It will be interesting to determine the developmental stages during which the gut wall

is receptive to colonisation by EPCs. In addition, it is unclear at this point whether exogenous neurons can establish functional synapses. Future *in vivo* transplantation experiments into aganglionic gut should address this question.

Upon transplantation into wild-type and aganglionic gut, both foetal and postnatal EPCs increased in number and colonised regions of the host gut at relatively long distance from the grafting site, suggesting that EPCs and their progeny have the capacity to respond to migratory and proliferative signals present in the intact gut wall. In addition, foetal and postnatal EPC progeny differentiated into neurons and glial cells upon grafting into wild-type foetal gut. Similarly, neuronal differentiation of EPCs took place within the gut of RET-deficient embryos. However, we were unable to detect EPC-derived GFAP<sup>+</sup> cells in grafted aganglionic gut. The reasons for the absence of glial cell differentiation are currently unclear. It is possible that the endogenous neural crest-derived cells present in the gut of wild-type embryos provide critical diffusible or cell-cell contact signals that are necessary for glial cell differentiation. Such signals, which could either be derived from undifferentiated progenitors of enteric neurons or differentiated neurons themselves, are expected to be reduced or absent in RET-deficient embryos which lack all neural crest-derived cells in the gut. Consistent with this hypothesis is the observation that gliogenesis in colonies of EPCs *in vitro* follows extensive neuronal differentiation.

### Implication of EPC isolation for HSCR disease treatment

At present, the only definitive therapy for HSCR is surgical resection of the aganglionic gut segment and anastomosis of the residual bowel, which is generally successful in relieving the immediate consequences of intestinal obstruction (Swenson, 2002; Tsuji et al., 1999). However, this approach is associated with short- and long-term morbidity and mortality and in a relatively large fraction of patients severe dysmotility persists postoperatively and can last for years (Tsuji et al., 1999). It is currently unclear whether such peristaltic malfunction is due to the surgical procedure itself or results from neuronal deficits of the ganglionated gut associated with the primary cause of aganglionosis. Therefore, we wished to consider the possibility of restoring peristalsis of aganglionic gut segments by transplanting ENS progenitors capable of forming a functional enteric plexus. According to one scenario, such progenitors could be derived from a small apparently normoganglionic gut segment of an affected individual and autotransplanted into the aganglionic gut region. Prerequisites for such procedure would be that (a) multipotential ENS progenitors could be derived from small gut segments (possibly the size of a biopsy sample), and (b) such progenitors could also be generated from the normoganglionic bowel segment of an HSCR patient. We have used wild-type mice and an animal model of HSCR to address both issues. Our findings show that it is feasible to isolate neural crest-derived progenitors capable of generating enteric neurons and glia. In addition, it is likely that such progenitors can be generated from relatively small gut segments of foetal and postnatal gut. Although in the present study we did not attempt to define the smallest possible gut segment that could generate sufficient numbers of EPCs, we have isolated EPCs from whole as well as segments of E11.5 guts (Fig. 10 and data not shown). This

together with the ability of EPCs to self-renew and proliferate suggests that it is feasible to isolate EPCs from relative small segments of human gut.

Although the majority of HSCR patients are characterised by regional aganglionosis which is usually restricted to the distal colon (Chakravarti, 2001), our recent analysis of mouse models with a similar phenotype indicated that, in addition to the colon, neurogenesis in the small intestine is also affected during embryogenesis. In embryos homozygous for mutations of *Ret* or *Et-3* (which encode components of the two signalling pathways most commonly affected in familial cases of HSCR) (Chakravarti, 2001) we observed reduced number and delayed migration of ENCCs prior to the arrival of these cells in the colon (Natarajan et al., 2002) (Barlow et al., 2003). Although these cellular deficits were apparently corrected throughout the small intestine and aganglionosis was eventually restricted to the colon, the possibility arises that the normoganglionic regions of the gut harbour subtle deficits. This hypothesis is further supported by a recent report indicating changes in neurotransmitter expression and the number of enteric neurons in the ileum of *Et-3<sup>ls/ls</sup>* animals (Sandgren et al., 2002). Such defects could provide a potential explanation for the peristaltic dysregulation observed postoperatively in patients that have undergone removal of the aganglionic gut segment and anastomosis. In addition, they raise the question as to whether the genetic deficits associated with aganglionosis preclude the efficient isolation of multipotential progenitors that could potentially be used to restore the neuronal plexus and the peristaltic activity of the affected gut region. Our experiments indicate that EPCs can be isolated from the apparently normoganglionic segment of the gut of *miRet<sup>51/51</sup>* embryos and neonates with efficiencies similar to those observed in wild-type embryos. Furthermore, in clonogenic assays such mutant EPCs were capable of generating both neuronal and glial cells. However, it is presently unclear whether *miRet<sup>51/51</sup>* EPCs are capable of generating the whole range of neuronal and glial subtypes encountered in similar cultures from wild-type EPCs. Also, it remains to be seen whether *miRet<sup>51/51</sup>* EPCs are capable of recolonising wild-type or aganglionic gut upon transplantation into intact gut in vitro or in vivo. The availability of the EPC clonogenic assay and gut transplantations will allow us to examine these possibilities and determine the neurogenic and gliogenic potential of multipotential progenitors from *miRet<sup>51/51</sup>* animals in vitro and in vivo.

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