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Maintenance of mammalian enteric nervous system progenitors by SOX10 and endothelin 3 signalling

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The transcriptional regulator SOX10 and the signalling molecule endothelin 3 have important roles in the development of the mammalian enteric nervous system (ENS). Using a clonal cell culture system, we show that SOX10 inhibits overt neuronal and glial differentiation of multilineage ENS progenitor cells (EPCs), without interfering with their neurogenic commitment. We also demonstrate that endothelin 3 inhibits reversibly the commitment and differentiation of EPCs along the neurogenic and gliogenic lineages, suggesting a role for this factor in the maintenance of multilineage ENS progenitors. Consistent with such a role, the proportion of *Sox10*-expressing progenitors in the total population of enteric neural crest cells is reduced in the gut of endothelin 3-deficient embryos. This reduction may be related to the requirement of endothelin signalling for the proliferation of ENS progenitors. The dependence of ENS progenitors on endothelin 3 is more pronounced at the migratory front of enteric neural crest cells, which is associated with relatively high levels of endothelin 3 mRNA. Our findings indicate that SOX10 and endothelin 3 have a crucial role in the maintenance of multilineage enteric nervous system progenitors.

KEY WORDS: Mouse, SOX10, Endothelin 3

INTRODUCTION

Elucidating the mechanisms by which extracellular signals and nuclear transcription factors coordinately control the development of the nervous system is crucial for understanding the pathogenesis, and devising strategies for the treatment, of neural deficiencies. Hirschsprung's disease (HSCR; congenital megacolon) is the most common form of congenital intestinal obstruction (1:4500 live births), which results from failure of enteric ganglia to develop in the distal bowel (colonic aganglionosis) (Chakravarti, 2001; Swenson, 2002). The familial form of HSCR has been associated mainly with mutations in *RET* (Brooks et al., 2005; Chakravarti, 2001), a locus encoding a tyrosine kinase receptor for members of the GDNF family of ligands (Baloh et al., 2000; Saarma, 2000). However, other genes, such as *SOX10* and *EDNRB*, have also been implicated in the pathogenesis of congenital megacolon (Amiel and Lyonnet, 2001; Brooks et al., 2005). Thus, haploinsufficiency for *SOX10*, which encodes an *SRY*-related HMG transcription factor (Wegner, 1999), is responsible for a significant proportion of cases of HSCR, primarily those associated with Waardenburg syndrome (Waardenburg-Hirschsprung's disease or Shah-Waardenburg syndrome), which, in addition to colonic aganglionosis, is characterised by deafness and hypopigmentation (Inoue et al., 2004; Pingault et al., 1998; Read and Newton, 1997). Consistent with the phenotype of *SOX10* mutations in humans, hemizygocity of this locus in mice is associated with aganglionosis of the terminal colon and white spotting, while animals homozygous for null mutations of *Sox10* have severe deficits of the entire peripheral nervous system (PNS) (Britsch et al., 2001; Herbarth et al., 1998; Kapur, 1999; Southard-Smith et al., 1998). The cellular and molecular mechanisms underlying such deficits are not clear, but studies on mouse embryos heterozygous for a targeted deletion of *Sox10* (*Sox10*^{LacZ}) have suggested that decreased levels of SOX10 result in a smaller ENS progenitor pool (Britsch et al., 2001; Paratore et al., 2002). By contrast, SOX10 overexpression in neural crest stem cells (NCSCs) allows them to maintain their neurogenic and gliogenic potential (Kim et al., 2003). *EDNRB*, which encodes a G protein-coupled receptor for endothelin 3 (EDN3), is also necessary for normal development of the ENS and is responsible for ~5% of cases of familial HSCR (Amiel and Lyonnet, 2001; Chakravarti, 2001; Edery et al., 1996; Hofstra et al., 1996; McCallion and Chakravarti, 2001; Puffenberger et al., 1994). The mechanisms by which the EDN3/EDNRB signalling pathway regulates enteric neurogenesis are currently unclear. Studies with mixed ENS cultures have shown that EDN3 inhibits neuronal differentiation (Hearn et al., 1998; Wu et al., 1999) and has a permissive effect on the proliferation of ENS progenitors (Barlow et al., 2003). However, other experiments have suggested that EDN3 signalling impairs the self-renewal of ENS progenitors by promoting their differentiation into myofibroblasts (Kruger et al., 2003).

Cultures of multilineage neural crest cell progenitors isolated from avian and mammalian embryos have served as valuable in vitro systems to study the role of extracellular signals and intracellular transcription factors in cell commitment and differentiation in the PNS (Cohen and Konigsberg, 1975; Dupin and Le Douarin, 1995; Dupin et al., 2001; Ito et al., 1993; Morrison et al., 2000; Sieber-Blum and Cohen, 1980; Stemple and Anderson, 1992). Multilineage progenitors of the ENS have also been isolated from the gut of rat and mouse embryos. Thus, NCSCs have been identified in the gut of rat embryos and postnatal animals using immunostaining for cell surface markers and fluorescence-activated cell sorting (FACS) (Bixby et al., 2002; Kruger et al., 2002). Enteric NCSCs generate colonies containing neurons, glia and myofibroblasts, while they colonise various PNS ganglia upon

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grafting into the migratory path of neural crest cells in avian embryos (Kruger et al., 2002). More recently, we have reported the isolation and characterisation of multilineage ENS progenitors from cultures of dissociated embryonic and postnatal mouse gut. Under appropriate conditions, such cultures generate neurosphere like bodies (NLBs), which are composed of differentiated cells as well as multipotential EPCs (ENS progenitor cells), which upon re-plating generate colonies containing enteric neurons and glia (Bondurand et al., 2003). EPCs represent pre-enteric vagal neural crest cells expressing *Sox10* and *Phox2b* but lack early neurogenic markers, such as MASH1 and RET. However, upon commitment to the neurogenic lineage, EPC progeny induce MASH1 and RET and differentiate into neurons which downregulate *Sox10* and *Mash1* (Ascl1 – Mouse Genome Informatics) but further upregulate *Ret* (Bondurand et al., 2003). Glial cells can also be identified in EPC-derived colonies subsequent to neuronal differentiation (Bondurand et al., 2003).

Here, we have used clonogenic cultures of EPCs to examine the role of SOX10 and endothelin signalling in the commitment and differentiation of multilineage ENS progenitors. In addition, we have analysed the ENS progenitor pool size, proliferation and differentiation in wild-type and EDN3-deficient mouse embryos. Our experiments indicate that SOX10 and EDN3 control the commitment and differentiation of ENS progenitors into enteric neurons and glia.

MATERIALS AND METHODS

Animals

Wild-type foetal and postnatal gut was isolated from Parkes (outbred) mice. Where indicated, guts were isolated from E11.5 $Sox10^{+/lacZ}$ (Britsch et al., 2001), $Ret^{51/51}$ (de Graaff et al., 2001) and $Ednrb^{LacZ/LacZ}$ (Lee et al., 2003) embryos. Tissue from wild-type littermates was used as control. Mice carrying the $Edn3^{ls}$ allele (Lyon, 1996) were obtained from the Medical Research Council Mammalian Genetics Unit (Harwell, UK) and backcrossed to C57Bl/6 mice for at least six generations. Heterozygous and homozygous $Edn3^{ls}$ animals were identified by PCR (Rice et al., 2000). The generation of $Tg^{Wnt1Cre}$ and $R26^{YFPStop}$ animals have been described previously (Danielian et al., 1998; Srinivas et al., 2001). The day of vaginal plug detection was considered to be E0.5.

Dissociated gut cultures and generation of EPCs

Generation of NLBs from dissociated gut cultures, isolation of EPCs and clonogenic cultures of EPCs have been described previously (Bondurand et al., 2003). Endothelin 3 (EDN3) (Calbiochem) was added to the culture medium at either 100 nM (dissociated gut cultures for NLB formation) or 10 nM (clonal cultures of FACS isolated EPCs). BQ788 (inhibitor of EDNRB signalling; Calbiochem) was used at a concentration of 100 nM. Cultures were re-fed every 2 days.

Acute cultures of dissociated intestine

Intestines were dissected from E11.5-E12.5 mouse embryos and incubated with dispase/collagenase (0.5 mg/ml in Phosphate Buffered Saline –1×PBS; Roche) for 3 minutes at room temperature. Tissue was washed three times in 1×PBS, dissociated into single cells by pipetting and plated onto fibronectin-coated (20 μg/ml) SONIC-SEAL slide wells (VWR) in optiMEM (Life Technologies) supplemented with L-glutamine (1 mM; Life Technologies) and penicillin/streptomycin antibiotic mixture (Life Technologies). Cultures were maintained for up to 3 hours prior to fixation in an atmosphere of 5% CO_2.

Retrovirus generation and infection

Details for the retrovirus generation and transduction have been reported previously (Bondurand et al., 2003). The SOX10-GFP retroviral transgene was generated by subcloning the human SOX10 cDNA (Bondurand et al., 2000) into the *Eco*RI and *Xho*I cloning sites of the pMX-IRES-GFP vector (Kitamura, 1998).

BrdU incorporation

BrdU (Sigma) stock solution (10 mg/ml) was made in 0.9% sodium chloride (Sigma). This solution was injected intraperitoneally (10 μl/g of animal weight) into pregnant mice and embryos were harvested 1 hour after the BrdU injection. Whole guts were dissected, fixed for 2 hours in 4% paraformaldehyde (PFA; in 1×PBS) at 4°C and immunostained as described below. Alternatively, embryonic intestines from BrdU-injected pregnant animals were dissociated into single-cell suspension, plated and maintained in optiMEM for 2-3 hours (acute cultures).

Immunostaining

Clonal cultures of EPCs were fixed in 4% PFA for 10 minutes at room temperature. After washing twice in PBS + 0.1% Triton X-100 (PBT), cells were incubated with blocking solution (PBT + 1%BSA + 0.15% glycine) at 4°C (overnight) or at room temperature (for 2-3 hours). Primary antibodies were diluted in blocking solution as follows: TuJ1 (mouse; BABCO, UK), 1:1000; GFP/YFP (mouse or Rabbit; Molecular Probes), 1:1000; RET (rabbit; Immuno-Biological Labs, Japan), 1:50; GFAP (rabbit; DAKO, USA), 1:400; B-FABP (rabbit; kind gift from Thomas Muller), 1:1000; SOX10 (mouse; kindly provided by Dr David Anderson), 1:10; SOX10 (rabbit; Chemicon), 1:200; MASH1 (mouse; kindly provided by Dr D. Anderson), 1:1; SMA (mouse clone 1A4; Sigma), 1:500; and MITF (mouse; Stratech), 1:200. Cultures were incubated with primary antibodies at room temperature (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 room temperature at the following dilutions: anti-mouse FITC-conjugated (Jackson Labs), 1:500; anti-rabbit FITC-conjugated (Jackson Labs), 1:500; anti-mouse AlexaFluor (Molecular Probes), 1:500; anti rabbit AlexaFluor (Molecular Probes), 1:500.

For immunostaining of acute cultures of intestines from embryos of BrdU-injected pregnant females, cells were fixed for 10 minutes in 4% PFA at 4°C, washed three times in PBT (5 minutes at room temperature) and incubated in PBS + 10% heat inactivated sheep serum (HISS) (overnight at 4°C). Antibodies for SOX10 and GFP were used as above. Following incubation with antibodies, cultures were washed three times (5 minutes each at room temperature) in PBT, post-fixed in 4% PFA for 10 minutes and incubated in 2 M HCl at 37°C for 30 minutes. HCl was removed using 0.1 M Borate Buffer (three washes, 10 minutes each). Incubation with BrdU antibody (rat; Oxford Biotechnology, 1:20) was for 5 hours at room temperature in PBT + 10% HISS and secondary antibody was anti-rat Alexa Fluor 568 (Molecular Probes, 1:500), which was added for 5 hours at room temperature followed by three 1-hour washes in PBT. The cultures were then mounted in Vectashield.

For whole-mount immunostaining, guts were fixed in 4% PFA at 4°C for 2 hours, washed three times (1 hour each) in PBT at room temperature and placed into PBT containing 10% heat-inactivated sheep serum (HISS) at room temperature for 5 hours. The explants were then incubated overnight at 4°C with antibodies for GFP (rabbit from Molecular Probes; diluted 1:1000 in PBT + 10% HISS) or TuJ1 (mouse from BABCO, UK; diluted 1:1000 in PBT + 10% HISS). Following antibody incubation, explants were rinsed three times in PBT (1 hour each) and incubated with the secondary antibody in PBT for 5 hours at room temperature (anti-rabbit Alexa Fluor 568; Molecular Probes, 1:500 or anti-mouse FITC-conjugated; Jackson Labs, 1:500). For BrdU immunostaining, preparations were then washed three times in PBT, post-fixed in 4% PFA and washed again with PBT (three times for 1 hour each). Following these washes, explants were incubated with 2 M HCl for 30 minutes at 37°C and then treated with 0.1 M borate buffer (three times for 10 minutes at room temperature). Incubation with anti-BrdU antibody (rat; Oxford Biotechnology, 1:20) was overnight at 4°C, while incubation with secondary antibody (anti-rat Alexa Fluor 568; Molecular Probes, 1:500) was for 5 hours at room temperature. Preparations were mounted using Vectashield (Vector Laboratories) or Vectashield containing DAPI.

Differentiation at the migratory wavefront was analysed using an Axiophot Zeiss epifluorescence microscope. The number of differentiated cells, as identified by TuJ1 expression, was determined at the migration wave-front using two different methods. First, the 30 more advanced YFP+ cells at the front of migration in each gut were identified and then

scored for TuJ1 immunostaining. Second, 50 single YFP+ cells present within the migration wave-front of each gut were counted and their double immunoreactivlty for TuJ1 was determined. Both methods gave identical results presented in Fig. 7. Images were analysed using Metamorph software package (Universal Imaging). For the proliferation analysis at the front of migration, YFP and BrdU immunostained preparations were examined using a Bio-Rad confocal microscope. Optical sections of 0.5 μm were photographed using a 60× lens and individual YFP+ cells present within the migration wave-front were identified and scored for immunoreactivity for BrdU. A minimum of 50 cells were counted within each gut preparation. All figures were compiled using Adobe Photoshop 7 software. Statistical analysis was carried out using a *t*-test. Differences were considered to be significant if *P* value was less that 0.05.

RESULTS

SOX10 overexpression inhibits neuronal and glial differentiation of multilineage ENS progenitors

Previous studies have shown that SOX10 maintains the neural and glial potential of NCSCs and inhibits overt neuronal differentiation in vitro and in vivo (Kim et al., 2003; McKeown et al., 2005). To examine the role of SOX10 in the differentiation of ENS progenitors, we used retroviral vectors to introduce either GFP or SOX10-IRES-GFP (SOX10-GFP) transgenes into clonogenic cultures of E11.5 EPCs (Bondurand et al., 2003) and determined the percentage of neurons and glial cells present in 5, 7 or 10 day-old colonies. We observed that in SOX10-GFP-expressing colonies, the percentage of neurons was significantly reduced relative to GFP controls (Fig. 1A-C). Despite this reduction, MASH1 and RET immunostaining was detected in a similar number of cells in GFP and SOX10-GFP colonies 5 days after plating, suggesting that SOX10 function does not interfere with the commitment of EPCs to the neuronal lineage (Fig. 1D-K). The effect of SOX10 overexpression on neuronal differentiation was unlikely to be a consequence of selective cell death, as the plating efficiency of the founder cells and the size of colonies generated by control and SOX10-GFP-expressing EPCs were similar (data not shown). In addition to neuronal differentiation, glial differentiation, as assessed by immunostaining for glial fibrillary acidic protein (GFAP), was also reduced in day-10 SOX10-GFP colonies relative to those expressing GFP alone (Fig. 1L,M). These findings indicate that the effects of SOX10 overexpression on EPC differentiation are similar to those observed in NCSCs (Kim et al., 2003) and suggest that one of the functions of this transcriptional regulator is to maintain the neurogenic and gliogenic potential of multilineage ENS progenitors.

Endothelin 3 signalling regulates neuronal and glial differentiation of EPCs

A potential mechanism by which SOX10 inhibits neuronal and glial differentiation of EPCs is the regulation of expression of a receptor(s) that, upon activation by its cognate ligand, maintains the undifferentiated state of these cells. In light of this, it is interesting that SOX10 regulates the expression of EDNRB (Zhu et al., 2004), which, upon activation by EDN3, inhibits neuronal differentiation in mixed ENS cultures (Hearn et al., 1998; Wu et al., 1999). Here, we have examined the potential role of endothelin signalling in the maintenance of multilineage ENS progenitors by analysing the effects of EDN3 on the differentiation of clonogenic EPC cultures. For this, EPCs derived from embryonic (E11.5) mouse gut were

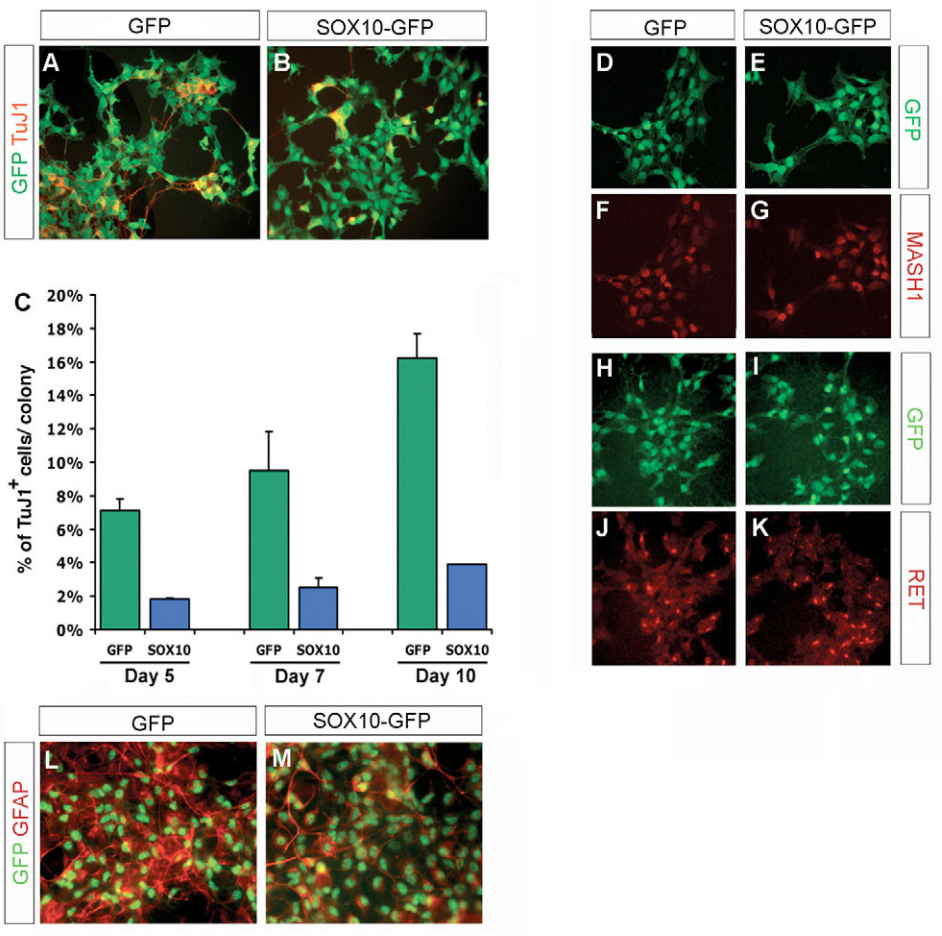

Fig. 1. Overexpression of SOX10 inhibits overt neuronal and glial differentiation of EPCs. (**A**,**B**) Staining of wild-type EPC colonies expressing either GFP (A) or SOX10-GFP (B) transgenes with antibodies for GFP (green) and TuJ1 (red). (**C**) Percentage of TuJ1$^+$ cells present in GFP- (green bars) or SOX10-GFP-(blue bars) expressing EPC colonies 5, 7 and 10 days after plating. (**D-K**) Day 5 EPC colonies expressing GFP (D,F,H,J) or SOX10-GFP (E,G,I,K) transgenes were immunostained for GFP (D,E,H,I), MASH1 (F,G) or RET (J,K). Optic fields in D,E and H,I are the same as those shown in F,G and J,K, respectively. (**L**,**M**) Day 10 GFP or SOX10-GFP colonies double immunostained for GFP (green) and GFAP (red).

plated at clonal densities and then allowed to form colonies in standard culture medium or in medium supplemented with EDN3 (10 nM). As expected, an increasing number of TuJ1$^+$ cells were observed in EPC colonies maintained in standard (control) medium (CM) for 5 to 10 days (Fig. 2A,C,G). By contrast, colonies forming in the presence of EDN3 had either no or very few neurons (Fig. 2B,D,G). The differentiation inhibiting effect of EDN3 was also evident in clonogenic cultures of EPCs derived from postnatal (PN) day 10 mouse gut (Fig. 2E,F). Unlike the constitutive expression of SOX10, endothelin signalling prevented induction of *Mash1* and *Ret* in EPC progeny (Fig. 2H-K). Thus, control day 5 colonies contained 24±7.4% and 26.7±9.4% of MASH1$^+$ and RET$^+$ cells respectively, but in the presence of EDN3 (10 nM) very few cells expressed these markers (0.8±0.3% and 2.5±0.7%, respectively) for at least 10 days. *Mash1* and *Ret* were not induced even after 10 days in the presence of BMP2, a factor known to have a neurogenic effect on NCSCs and EPCs [(Kim et al., 2003; Shah et al., 1996) and data not shown]. EDN3 also blocked glial differentiation in clonogenic cultures of EPCs. This is indicated by the dramatic reduction among the progeny of embryonic and postnatal EPCs of cells expressing the glial-specific markers brain-specific fatty acid binding protein (B-FABP) (Kurtz et al., 1994; Young et al., 2003) on day 5 or GFAP on day 10 (Fig. 2L-Q). In EPC colonies maintained in control medium, expression of *Sox10* is normally downregulated in enteric neurons but maintained in glial cells (Bondurand et al., 2003). Despite the inhibition of glial differentiation, the vast majority of cells (at least 95%) in EPC colonies maintained in EDN3-supplemented medium maintained high levels of SOX10, indicating their undifferentiated progenitor status (Fig. 2R-U,V). Addition of EDN3 to our standard culture medium generally did not alter colony size. Moreover, immunostaining for activated caspase 3 revealed no difference in the extent of apoptosis detected in the presence (2.2±2.0%) or absence (2.3±2.2%) of EDN3. Thus, EDN3 inhibits neuronal and glial differentiation of EPCs but this differentiation-inhibiting effect is unlikely to result from selective elimination of differentiated cells.

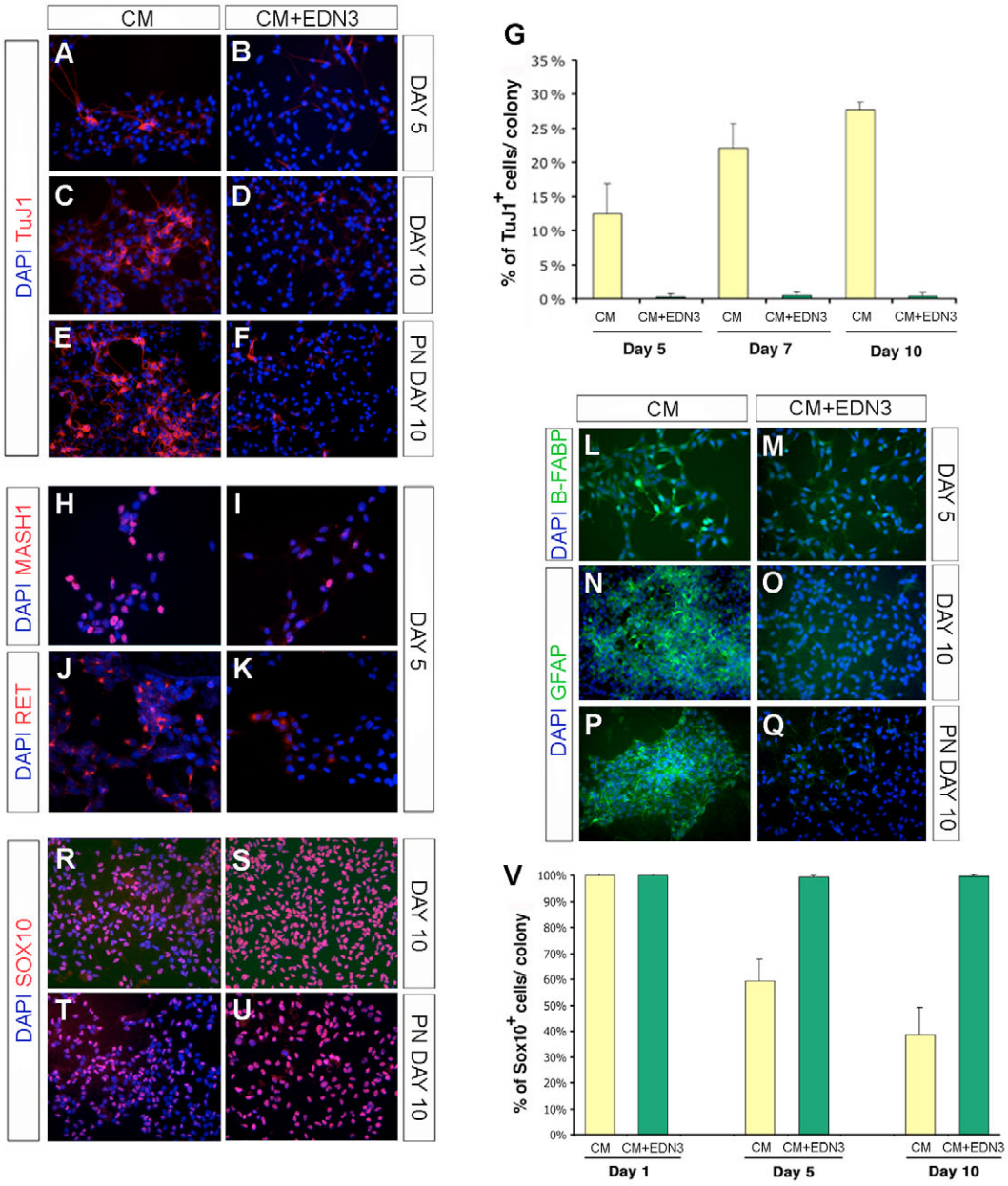

Fig. 2. EDN3 signalling inhibits lineage commitment and differentiation of EPCs. Wild-type EPCs were isolated from cultures of embryonic (A-D,H-K,L-O,R,S) or postnatal (E,F,P,Q,T,U) gut and maintained in either standard (control) medium (CM; A,C,E,H,J,L,N,P,R,T) or CM supplemented with 10 nM EDN3 (B,D,F,I,K,M,O,Q,S,U). Colonies were fixed 5 (A,B,H-K,L,M) or 10 (C-F,N-Q,R-U) days after plating and immunostained for TuJ1 (**A-F**), MASH1 (**H**,**I**), RET (**J**,**K**), B-FABP (**L**,**M**), GFAP (**N-Q**) and SOX10 (**R-U**). In all panels, colonies were counterstained with DAPI. (**G**) Percentage of TuJ1$^+$ cells in colonies maintained either in standard medium (CM; yellow bars) or medium supplemented with 10 nM EDN3 (CM+EDN3; green bars) 5, 7 and 10 days after plating. (**V**) Percentage of SOX10$^+$ cells in colonies maintained in CM (yellow bars) or CM+EDN3 (green bars) 1, 5 or 10 days after plating.

Next we considered the possibility that the anti-neurogenic and anti-gliogenic effects of EDN3 are due to the irreversible commitment of EPCs to alternative cell lineages. This was prompted by experiments indicating that EDN3 promotes differentiation of rat embryo gut-derived NCSCs into smooth muscle actin-positive (SMA^+) myofibroblasts (Kruger et al., 2003). To explore the possibility that endothelin signalling drives differentiation of mouse ENS progenitors towards cell types that lack neurogenic and gliogenic potential, EPC colonies forming in the presence of EDN3 were immunostained for SMA and MITF [a marker of neural crest-derived melanocytes (Widlund and Fisher, 2003)]. Neither of these markers was detected in colonies maintained for up to 15 days in the presence or absence of EDN3 (Fig. 3A-F), suggesting that irreversible differentiation of EPCs into myofibroblasts or melanocytes may not account for the lack of neuronal and glial differentiation.

To further address this issue, we also examined whether the differentiation-inhibiting effect of EDN3 on EPC colonies was reversible. For this, freshly isolated EPCs were cultured in the presence of EDN3 for up to 7 days and then transferred to EDN3-free medium supplemented with BQ788 (a specific inhibitor of EDNRB used here to block residual EDN3 activity), for an additional 5 days. As expected, colonies maintained in standard medium throughout the culture period (up to 12 days) contained many neurons and glia (Fig. 3G-I), while in the presence of EDN3, the percentage of both cell types was drastically reduced (Fig. 3G,J,K). Interestingly, removal of endothelin signalling 5-7 days after plating resulted in significant recovery of neuronal and glial differentiation (Fig. 3G,L-O). Taken together, our studies suggest that EDN3 inhibits reversibly the commitment and differentiation of multipotent ENS progenitors along the neuronal and glial lineages, and that this effect is unlikely to result from the early commitment of EPCs to an irreversible non-neurogenic or non-gliogenic differentiation pathway.

Normal Sox10 activity and endothelin signalling are required for the formation of NLBs and EPCs in dissociated gut cultures

We have previously suggested that formation of NLBs and recovery of EPCs from dissociated gut cultures depends on the self-renewing capacity of undifferentiated ENS progenitors (Bondurand et al., 2003). This idea is further supported by our present studies, demonstrating a significant reduction in the size and number of NLBs and EPCs generated from cultures of gut isolated from E11.5

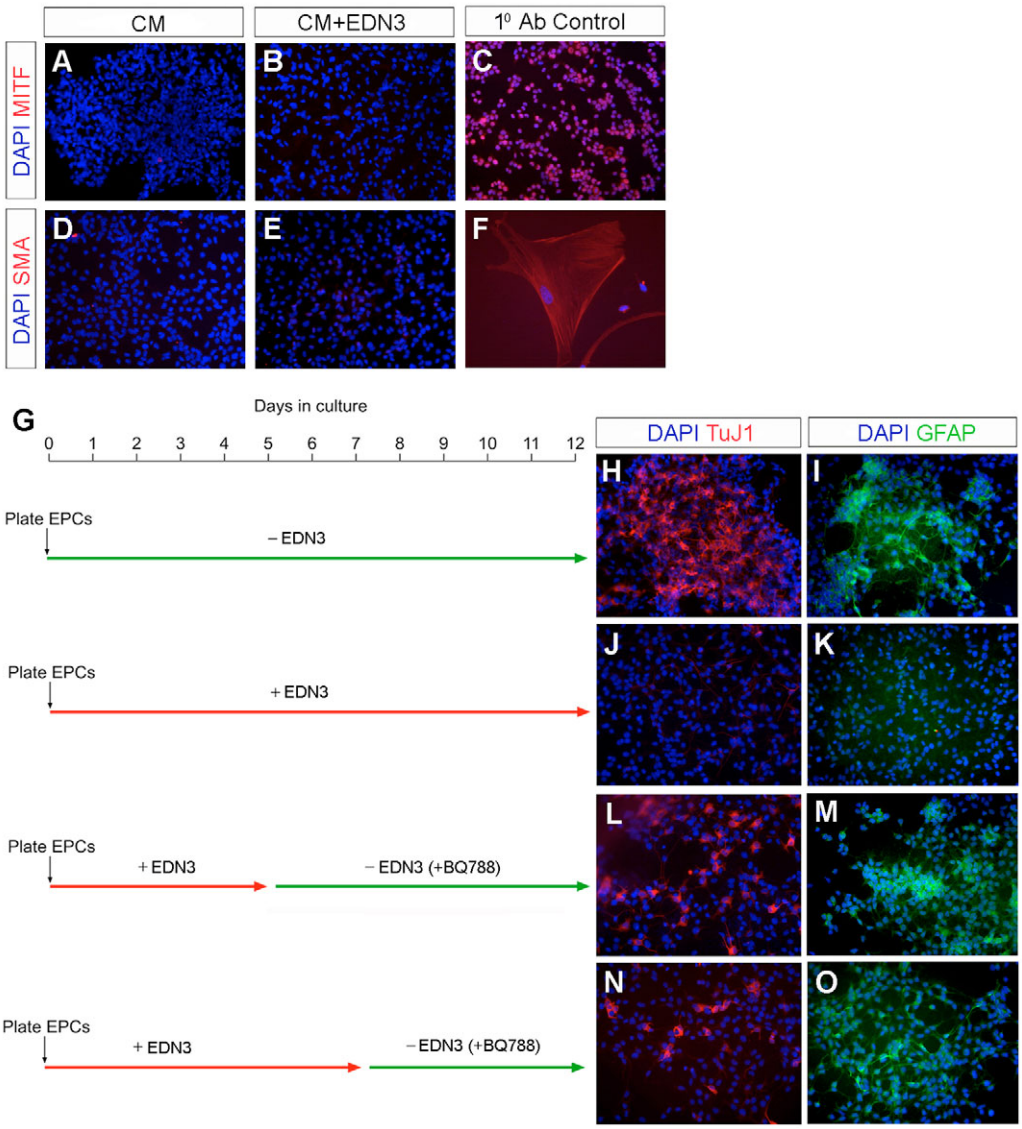

Fig. 3. The inhibitory effect of EDN3 on lineage commitment and differentiation of EPCs is reversible. Colonies from embryonic EPCs (**A**,**B**,**D**,**E**) were maintained in standard medium (CM; A,D) or standard medium supplemented with 10 nM EDN3 (CM+EDN3; B,E) and immunostained for MITF (A,B) or SMA (D,E). (**C**,**F**) Images of cultured SkMe15 cells known to express MITF [American Type Culture Collection, ATCC (Du et al., 2003)] (C) and a cell in a primary embryonic gut culture that expresses SMA (F). (**G**) The culture conditions of freshly isolated (day 0) EPCs. At the end of the culture period (day 12) colonies in each condition were fixed and immunostained for TuJ1 (**H**,**J**,**L**,**N**) or GFAP (**I**,**K**,**M**,**O**). All colonies were counterstained with DAPI.

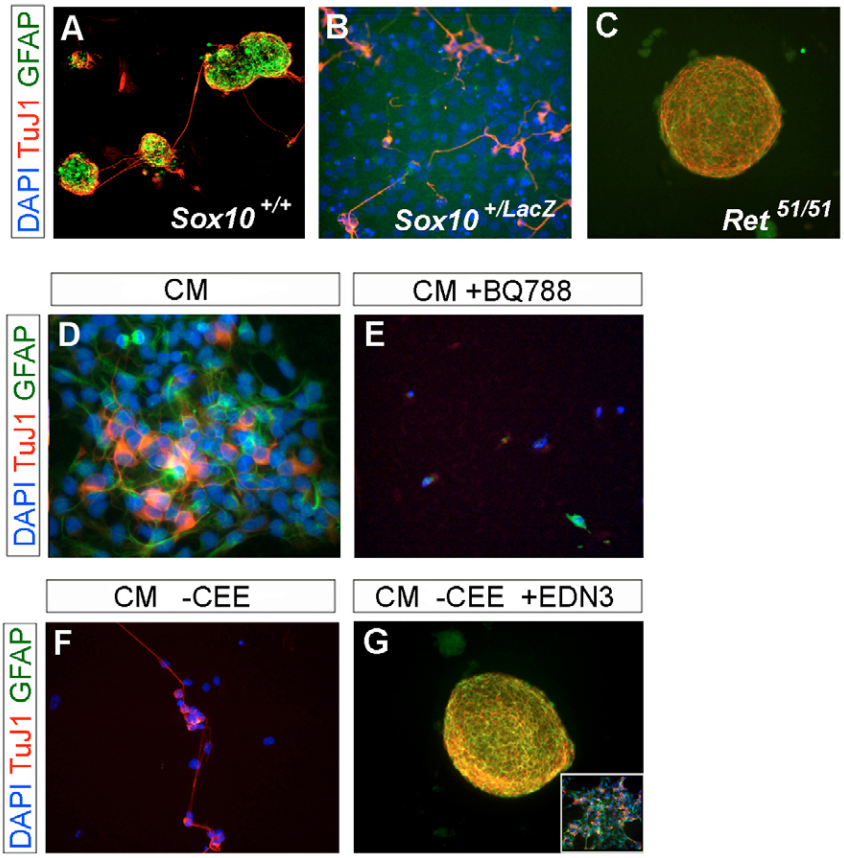

Fig. 4. Normal SOX10 activity and EDN3 signalling are required for formation of NLBs and EPCs. Guts from E11.5 wild-type (**A**), *Sox10*$^{+/LacZ}$ (**B**) or *Ret*$^{51/51}$ (**C**) embryos were dissociated and cultured under conditions that promote the formation of NLBs. After fixation, cultures were immunostained for TuJ1 (red) and GFAP (green), and counterstained with DAPI. NLBs form readily in gut cultures from wild-type and *Ret*$^{51/51}$ embryos but fail to form in cultures from embryos hemizygous for *Sox10*. In wild-type gut cultures maintained in standard medium (CM), NLBs form characteristic colonies composed of undifferentiated cells, neurons and glia (Bondurand et al., 2003). Such a colony immunostained for TuJ1 (red) and GFAP (green) is shown (**D**). These characteristic colonies do not appear in the presence of BQ788 (**E**). In the absence of CEE, embryo gut cultures failed to form NLBs (**F**). However, addition of EDN3 was sufficient to rescue the formation of NLBs (**G**) and EPCs capable of generating multilineage colonies. Part of such an EPC colony is shown in the inset of G.

embryos hemizygous for a deletion of *Sox10* (*Sox10*$^{+/LacZ}$) (Britsch et al., 2001), relative to similar cultures from wild-type littermates (Fig. 4A,B). This deficit does not appear to reflect the reduced number of neural crest cells in the gut of *Sox10*$^{+/LacZ}$ embryos (Paratore et al., 2002), as parallel experiments with similar stage embryos homozygous for *Ret*^{51}, which have a severe reduction in the number of enteric neural crest cells [(de Graaff et al., 2001); D.N. and V.P., unpublished], yield normal sized and numbers of NLBs and EPCs [Fig. 4C; see also Bondurand et al. (Bondurand et al., 2003)]. Therefore, the effect of *Sox10* hemizygocity on NLB and EPC formation is specific and reflects the requirement of this transcriptional regulator in the maintenance and propagation of multilineage ENS progenitors.

Next, we examined the role of EDN3 signalling in the formation of NLBs and EPCs in embryonic gut cultures. We observed that cultures maintained in the presence of BQ788 failed to generate the characteristic colonies-harbingers of NLBs (Bondurand et al., 2003) and NLBs themselves (Fig. 4D,E; data not shown). Consistent with a role of endothelin signalling in NLB formation, NLBs and EPC formation was drastically reduced in gut cultures from embryos homozygous for a targeted deletion of *Ednrb* (Lee et al., 2003) (data not shown). These findings indicate that formation of NLBs and generation of EPCs in dissociated gut cultures requires EDN3 signalling. As no purified endothelin is normally added to the culture medium, the putative source for this (or a related) factor is the chicken embryo extract (CEE) supplement (Bondurand et al., 2003; Morrison et al., 1999; Stemple and Anderson, 1992). Consistent with this idea, NLBs and EPCs fail to form in cultures maintained in CEE-free medium (Fig. 4F). However, addition of EDN3 (100 nM) to CEE-free medium was sufficient to rescue the formation of both NLBs and EPCs (Fig. 4G). Taken together, these experiments suggest that normal activities of SOX10 and EDN3/EDNRB, are required for the propagation and maintenance of multilineage ENS progenitors in dissociated gut cultures.

Reduced size of the pool of ENS progenitors in EDN3-deficient animals

In a recent study, Paratore and colleagues have shown that the colonic aganglionosis of *Sox10* haploinsufficient animals (*Sox10*$^{+/LacZ}$) is associated with depletion of ENS progenitors (identified by expression of *Sox10*) in the midgut of mutant embryos (Britsch et al., 2001; Paratore et al., 2002). To examine directly whether absence of EDN3 signalling results in a similar reduction in the size of the ENS progenitor pool, we determined the fraction of *Sox10*-expressing cells within the total neural crest cell population in the gut of wild-type and EDN3-deficient E11.5-E12.0 mouse embryos. At this stage, overt glial differentiation is not apparent, and expression of *Sox10* marks multilineage ENS progenitors (Paratore et al., 2002; Young et al., 2003) (N.B., D.N., A.B., N.T. and V.P., unpublished). To perform this analysis, we first established an in vivo lineage marking system, which allowed us to identify unambiguously the relatively small population of neural crest-derived cells within the gut of mouse embryos. This system is based on the generation of animals carrying a *Wnt1-Cre* transgene (*Tg*^{Wnt1Cre}), which encodes the bacterial Cre recombinase under the control of Wnt1 regulatory DNA sequences (Danielian et al., 1998), and the *R26*^{YFPStop} reporter allele, generated by inserting into the wild-type *Rosa26* (*R26*) locus, the coding sequence of yellow fluorescent protein preceded by loxP-flanked stop sequence (YFPStop) (Srinivas et al., 2001). As *Wnt1-Cre* is specifically expressed along the length of the dorsal neural tube and thus by all neural crest cells (Danielian et al., 1998) (S. Bogni and V.P.,

unpublished), $Tg^{Wnt1Cre}$;$R26^{YFPStop}$ animals express YFP in neural crest cells and their derivatives, including those colonising the embryonic gut (Fig. 5A). By carrying out the appropriate crosses, we next introduced the loss of function $Edn3^{ls}$ allele into the $Tg^{Wnt1Cre}$;$R26^{YFPStop}$ background. Fluorescent E11.5-E12.0 embryos derived by intercrossing mice heterozygous at each of the three loci (i.e. $Edn3^{ls}$, $Tg^{Wnt1Cre}$ and $R26^{YFPStop}$) were identified and their intestines were dissociated into single cell suspension and plated. Shortly after plating (at most 2-3 hours) the cultures were fixed and double immunostained for YFP and SOX10. The proportion of YFP$^+$ cells in such acute cultures established from $Edn3^{ls}$ homozygous embryos was decreased by ~65% relative to the equivalent percentage in wild-type embryos. Furthermore, in cultures established from $Edn3^{+/+}$ and $Edn3^{+/ls}$ embryos, SOX10$^+$ cells represented, respectively, 71.6±3.9% and 68.5±3.9% of the total neural crest-derived (YFP$^+$) cell population within the gut (Fig. 5B). By contrast, in the gut of $Edn3^{ls/ls}$ embryos, the SOX10$^+$YFP$^+$/YFP$^+$ fraction of cells was significantly reduced (56.2±5.8%; P<0.05, n=6; Fig. 5B). Using a similar approach, we have also observed that the proportion of SOX10$^+$ cells within the enteric neural crest cell lineage was reduced by 20% in embryos homozygous for a targeted allele of *Ednrb* (Lee et al., 2003) relative to heterozygous or wild-type controls (data not shown). Therefore, severe deficit in EDN3 signalling leads to a relative reduction in the pool of *Sox10*-expressing ENS progenitors within the gut of mouse embryos.

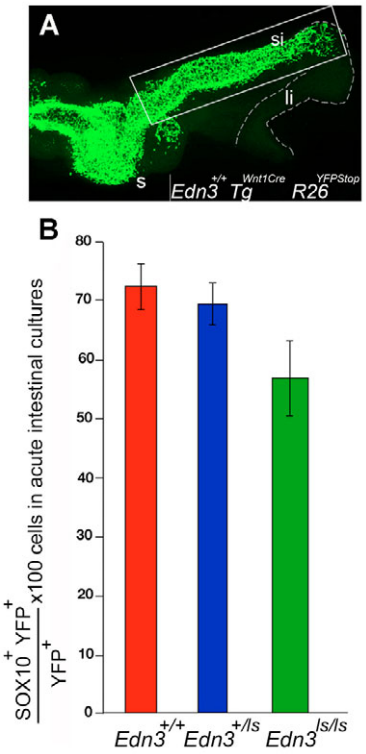

Fig. 5. Reduction of the pool of ENS progenitors in $Edn3^{ls/ls}$ embryos. (**A**) Whole-mount preparation of gut from $Edn3^{+/+}$; $Tg^{Wnt1Cre}$; $R26^{YFPStop}$ embryos immunostained for YFP. Box indicates the part of gut used to generate the intestinal acute cultures. (**B**) Bars represent the fraction of double positive (SOX10$^+$YFP$^+$) cells relative to the total population of neural crest-derived (YFP$^+$) cells in acute intestinal cultures established from embryos wild-type (red) or heterozygous (blue) and homozygous for $Edn3^{ls}$ (green).

Reduced proliferation of Sox10-expressing cells in the gut of EDN3-deficient embryos

The smaller fraction of SOX10$^+$ ENS progenitors within EDN3-deficient embryonic guts could result from reduced proliferation of this cell population. Consistent with this idea, we have previously observed a reduction in the mitotic fraction of SOX10$^+$ cells in the intestines of E11.5-E12.0 $Edn3^{ls}$ homozygous embryos (Barlow et al., 2003). However, other reports have indicated that EDNRB signalling is not required for the proliferation of enteric NCSCs of rat embryos (Kruger et al., 2003). Here, we have re-examined the role of EDN3 in the proliferation of endogenous ENS progenitors by analysing the incorporation of the nucleotide analogue bromodeoxyuridine (BrdU) into YFP$^+$ or SOX10$^+$ cells in the intestine of wild-type, $Edn3^{+/ls}$ and $Edn3^{ls/ls}$ embryos. For this, animals heterozygous for $Edn3^{ls}$, $Tg^{Wnt1Cre}$ and $R26^{YFPStop}$ were intercrossed and pregnant females (at E11.5) were injected with BrdU. One hour later, the intestines of individual embryos were dissociated, plated as acute cultures, and immunostained for BrdU and YFP or SOX10. We found that the fraction of BrdU$^+$YFP$^+$/YFP$^+$cells was similar in the intestine of wild-type, $Edn3^{+/ls}$ or $Edn3^{ls/ls}$ embryos (31.0±4.5%, 33.4±3.3% and 31.0±4.4%, respectively, n=5; Fig. 6A,C). In addition, a similar fraction of BrdU$^+$SOX10$^+$/SOX10$^+$ cells was found in the intestine of wild-type and $Edn3^{+/ls}$ E11.5 embryos (48.7±5.0% and 46.2±3.6%, respectively; Fig. 6D). By contrast, the fraction of BrdU$^+$SOX10$^+$/SOX10$^+$ cells was reduced in the gut of $Edn3^{ls/ls}$ embryos (39.9±4.8%) relative to wild-type or $Edn3^{+/ls}$ littermates (P<0.007, n=10; Fig. 6D). Therefore, severe reduction in EDN3 signalling results in a smaller fraction of proliferating SOX10$^+$ cells within the intestine of $Edn3^{ls}$ homozygous embryos.

The above experiments were designed to examine the global effect of the $Edn3^{ls}$ mutation on the proliferation of enteric neural crest-derived cells irrespective of their position along the length of the intestine. However, previous studies have demonstrated that expression of *Edn3* is spatially and temporally regulated during gut organogenesis, with highest levels detected in intestinal segments harbouring the front of rostrocaudally migrating neural crest cells. For example, in E11.5 embryos, the front of migrating neural crest cells is crossing the ileocecal valve to enter the cecum, while the highest levels of Edn3 mRNA are detected in the cecum and the proximal colon (Barlow et al., 2003; Leibl et al., 1999). These data raise the possibility that enteric neural crest cells at the migratory front are exposed to higher levels of EDN3 and thus may be more dependent on this signalling molecule for their proliferation. To examine this possibility, pregnant mice from $Edn3^{ls}$, $Tg^{Wnt1Cre}$ and $R26^{YFPStop}$ intercrosses were pulsed for 1 hour with BrdU, and the guts of individual fluorescent embryos (at E11.5) were double immunostained as whole-mount preparations with antibodies specific for BrdU and YFP and analysed by confocal microscopy (Fig. 6A,B). Contrary to the acute culture experiments, we observed a significant reduction in the fraction of BrdU$^+$YFP$^+$/YFP$^+$ cells at the migratory front of $Edn3^{ls/ls}$ embryos (48.9±6.7%) compared with wild-type littermates (61.8±5.0; P<0.007, n=6) (Fig. 6E). The equivalent value for $Edn3^{+/ls}$ animals was 51.8±8.9%, which indicated a trend towards reduced proliferation even in heterozygous embryos (Fig. 6E). The very high density of neural crest-derived cells behind the front of migration precluded a similar analysis to be carried out in more proximal gut regions. In addition, for technical reasons, we were unable to perform a similar analysis using a combination of SOX10- and YFP-specific antibodies. In summary, our experiments indicate that decreased endothelin signalling in vivo leads to a reduction in the proliferation of *Sox10*-expressing

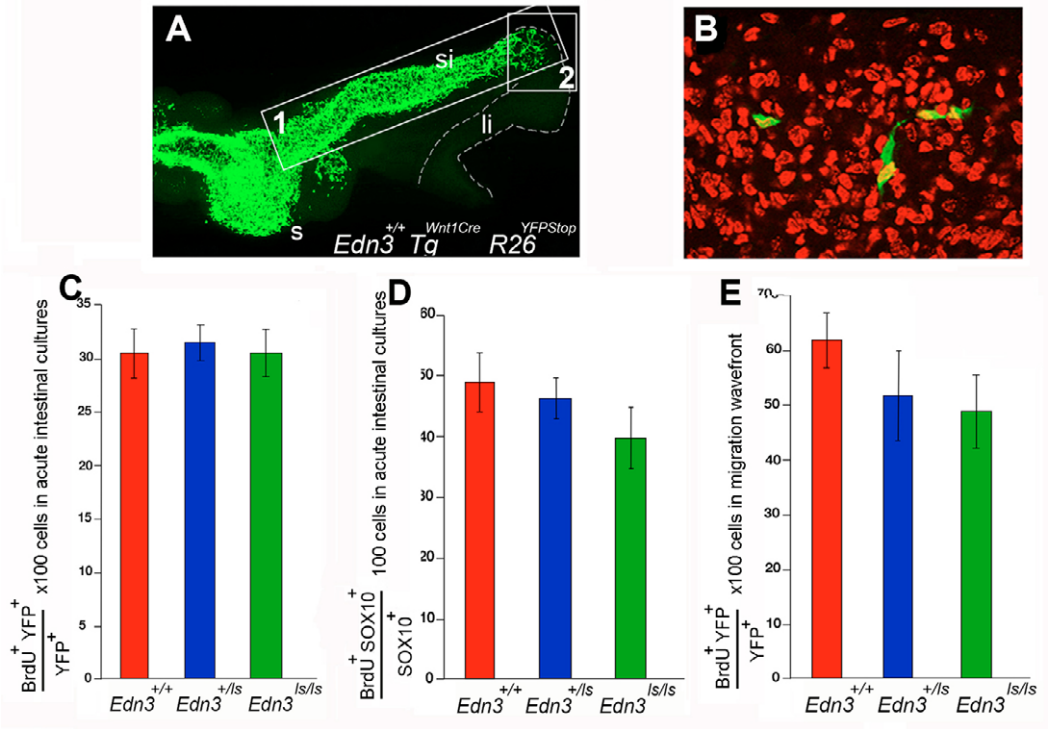

Fig. 6. Reduced EDN3 signalling decreases the proliferation of specific subpopulations of enteric neural crest cells. (**A**) Whole-mount preparation of gut from *Edn3*$^{+/+}$; *Tg*^{Wnt1Cre}; *R26*^{YFPStop} embryos immunostained for YFP. Box 1 indicates the part of gut used to generate the intestinal acute cultures examined in C and D. Box 2 indicates the area of the gut analysed in B and E. (**B**) Confocal image of an embryonic gut region corresponding to box 2 of A. Gut was immunostained for BrdU (red) and YFP (green). (**C**) Bars represent the fraction of BrdU^{+}YFP^{+} cells relative to the total population of neural crest-derived (YFP^{+}) cells in acute intestinal cultures from wild-type (red), *Edn3*$^{+/ls}$ (blue) and *Edn3*$^{ls/ls}$ (green) embryos. (**D**) Percentage of BrdU^{+}SOX10^{+} cells within the total population of SOX10^{+} cells in acute intestinal cultures from wild-type, *Edn3*$^{+/ls}$ and *Edn3*$^{ls/ls}$ embryos. (**E**) Fraction of BrdU^{+}YFP^{+} cells relative to the total population of YFP^{+} cells at the migratory wavefront of enteric neural crest cells.

multilineage ENS progenitors. This requirement for endothelin signalling appears to be spatially regulated along the intestine and is highest in neural crest cells at the front of migration.

Increased neuronal differentiation of ENS progenitors at the front of migration

Our findings raise the possibility that neural crest cells at the migratory front in the gut of *Edn3*$^{ls/ls}$ embryos exit the cell cycle and differentiate prematurely. To examine this possibility further, fluorescent guts from E11.5-E12.0 embryos generated by intercrossing animals heterozygous for *Edn3*^{ls}, *Tg*^{Wnt1Cre}, *Rosa26*^{YFPStop} were dissected and immunostained as whole-mount preparations for YFP and TuJ1 (Fig. 7A,B; see also Fig. 6A). In wild-type embryos, 6.5±2.8% of YFP^{+} cells at the front of migration (ileocaecal valve and cecum) were also positive for TuJ1 (YFP^{+}TuJ1^{+}). By contrast, the corresponding figures for embryos heterozygous or homozygous for the *Edn3*^{ls} allele were 9.0±2.4% and 13.0±2.5%, respectively (Fig. 7C). Therefore, in *Edn3*^{ls} homozygous mutants reduced neural crest cell proliferation at the migratory front is associated with an increased proportion of cells that have initiated neuronal differentiation.

To examine whether the increase in neuronal differentiation was restricted to the front of migration, we also determined the percentage of TuJ1-expressing cells in the entire intestinal population of YFP^{+} cells by establishing acute cultures of dissociated intestine dissected from E11.5-12.0 embryos. We found that the overall proportion of TuJ1^{+}YFP^{+}/YFP^{+} cells within the gut of *Edn3*$^{ls/ls}$ embryos was decreased relative to wild-type or heterozygous mutants (data not shown), indicating that severe deficit in EDN3 signalling reduces the overall differentiation of enteric neural crest cells.

DISCUSSION

Using clonogenic cultures of EPCs, we have examined the role of SOX10 and EDN3 signalling on the commitment and differentiation of multilineage progenitors of the mammalian ENS. Our experiments show that overexpression of SOX10 inhibits enteric neuron and glia differentiation without preventing commitment to the neurogenic lineage. Moreover, normal *Sox10* activity is required for the recovery of multilineage ENS progenitors (EPCs) from embryonic gut cultures. These findings, together with studies on *Sox10* hemizygous embryos (*Sox10*^{LacZ}) demonstrating a specific reduction in the pool of multipotential ENS progenitors (Paratore et al., 2002), indicate that SOX10 functions during embryogenesis to maintain and propagate the neurogenic and gliogenic progenitors of the ENS. Independent experiments have shown that overexpression of SOX10 in the dorsal neural tube can induce neural crest cells in avian embryos (McKeown et al., 2005), while studies on NCSCs have demonstrated that high levels of this factor are sufficient to maintain their neurogenic and gliogenic potential (Kim et al., 2003). Therefore, SOX10 appears to have overlapping roles within the neural crest cell lineage, contributing to its early induction in the neural tube and the maintenance of its multipotential state.

The inhibitory role of endothelin signalling on neuronal differentiation has been reported previously by two groups, who have shown that upon addition of EDN3 (or the EDNRB agonist

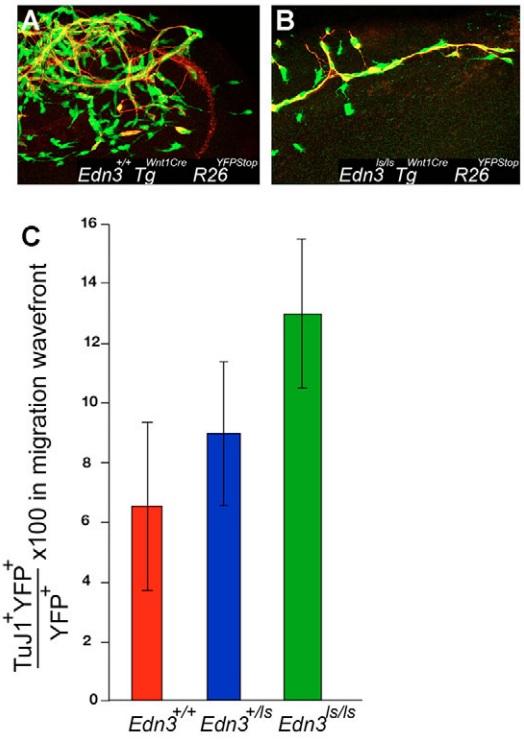

Fig. 7. Increased neuronal differentiation of enteric neural crest cells at the migratory front. (**A**,**B**) Whole-mount preparations of guts from E11.5 $Tg^{Wnt1Cre};R26^{YFPStop}$ embryos wild-type ($Edn3^{+/+}$; A) or homozygous for the *ls* allele ($Edn3^{ls/ls}$; B); guts were immunostained for YFP (green) and TuJ1 (red). Images correspond to the front of enteric neural crest cell migration (part of box 2 of the gut shown in Fig. 6A). (**C**) Bars represent the fraction of double-positive ($TuJ1^+YFP^+$) cells within the total population of YFP^+ cells present at the front of migration of wild-type (red), $Edn3^{+/ls}$ (blue) and $Edn3^{ls/ls}$ (green) embryos.

IRL1620), primary cultures of enteric neural crest-derived cells yield fewer postmitotic enteric neurons (Hearn et al., 1998; Wu et al., 1999). However, in these studies EDN3 and IRL1620 were added to a mixed population of neural crest-derived gut cells representing various stages of commitment and differentiation, thus precluding the identification of the precise cell target for endothelin. Here, we have employed the experimental paradigm of clonogenic EPC cultures to explore specifically the role of EDN3 in the commitment and differentiation of multilineage ENS progenitors. Our studies show that, similar to SOX10 overexpression, EDN3 inhibits overt neuronal and glial differentiation. However, unlike SOX10, EDN3 prevents commitment of EPCs to the neurogenic and gliogenic pathways. Taken together, these studies suggest that one of the roles of endothelin signalling during gut organogenesis is to maintain ENS progenitors in an uncommitted, multipotential and self-renewing state. This suggestion is further supported by our in vivo analysis of the intestine of E11.5-E12.0 mouse embryos homozygous for the $Edn3^{ls}$ mutation, which demonstrated a relative reduction of the pool of multipotential ENS progenitors.

Compared with the dramatic effect of EDN3 on the differentiation of EPC colonies, animals homozygous for $Edn3^{ls}$ show a relatively small decrease in the ENS progenitor pool (this study) and have restricted colonic aganglionosis (Lyon, 1996). The apparent discrepancy between the in vitro and in vivo effects of EDN3 could be explained by the presence within embryos of additional factors that can substitute for its absence. One such factor is EDN1, a peptide closely related to EDN3 (Hirata, 1996). However, expression of EDN1 or its receptor EDNRA has not been reported within the gut of mouse embryos and the potential effects of *Edn1* and *Ednra* mutations (Pla and Larue, 2003) on the development of the mammalian ENS are unclear. Other signalling pathways could also function in series or in parallel to EDN3 to regulate the number and differentiation of ENS progenitors in vivo. For example, the receptor tyrosine kinase RET is known to interact with the EDN3 signalling pathway to regulate the severity of the phenotype in individuals with HSCR and the extent of colonisation by neural crest cells of the gut of mouse embryos (Barlow et al., 2003; Carrasquillo et al., 2002; Kruger et al., 2003; McCallion et al., 2003). Marker analysis of vagal neural crest-derived cells in the gut of E9.0-E10.5 embryos and clonogenic cultures of EPCs, have indicated that the $SOX10^+RET^-$ population of pre-enteric neural crest cells that invades the foregut gradually induces expression of *Ret* and (in the case of neurogenic sublineage) eventually downregulates *Sox10* (Anderson et al., 2006; Bondurand et al., 2003; Young et al., 2003). Although absence of EDN3 during embryogenesis is expected to reduce the number of early multilineage ENS progenitors and thus the supply of RET^+ progeny, activation of RET by GDNF could partly compensate for this deficit by stimulating the proliferation of *Ret*-expressing descendants (Gianino et al., 2003; Heuckeroth et al., 1998). This idea is consistent with the severe ENS phenotype observed in embryos bearing mutations in both *Ret* and *Edn3* genes (Barlow et al., 2003), and suggests that EDN3 and GDNF control the size of partially overlapping cell populations within the gut.

Our experiments, together with other genetic studies (Cantrell et al., 2004), indicate that *Sox10* and *Edn3*/*Ednrb* are components of a signalling cascade that controls commitment and differentiation in the mammalian ENS. This idea is further supported by analysis of transgenic mice, demonstrating that *Sox10* regulates the spatial and temporal expression of *Ednrb* in neural crest cells (Zhu et al., 2004). Interestingly, other studies have implicated SOX10 in the regulation of expression of *Ret* in the ENS of mouse embryos (Lang et al., 2000; Lang et al., 2003). Therefore, *Sox10* activity appears to have antagonistic effects on enteric neural crest cells by promoting the expression of receptors associated with maintenance of cells in an uncommitted and undifferentiated state (EDNRB) but also commitment and differentiation towards mature phenotypes (RET). This apparent paradox may be explained by the specific challenges faced by neural crest cells upon invasion of the foregut. Thus, as these cells migrate to colonise the gut, the number of multilineage progenitors must remain sufficiently high for a relatively long period in order for the appropriate number of progeny to colonise an expanding gastrointestinal tube. Therefore, exposure of pre-enteric neural crest cells to differentiation-promoting signals from the splachnic mesenchyme of the gut must be initially neutralised by the employment of differentiation-inhibiting mechanisms that preserve a relatively high proportion of ENS progenitors in an uncommitted and undifferentiated state. Consistent with this hypothesis, arrival of pre-enteric neural crest cells to the vicinity of the aorta and the foregut is associated with induction of *Ret* [presumably under the influence of BMPs (Lo et al., 1997)], which, upon activation by GDNF, is capable of promoting neuronal differentiation (Taraviras et al., 1999). Under these conditions, EDN3/EDNRB signalling would be specifically required within the gut as an 'antidote' to the neurogenic and differentiation-inducing signals from the splachnic mesenchyme. In support of this idea, we have observed that the differentiation-promoting effect of BMPs and GDNF on EPC colonies can be blocked efficiently by EDN3 (N.B., D.N., A.B., N.T.

and V.P., unpublished). However, the commitment and differentiation-inhibiting effects of EDN3 on ENS progenitors must be transient and reversible, as neural crest progenitors must eventually escape the differentiation block to generate mature neuronal and glial phenotypes. The molecular mechanisms controlling the release from such an 'EDN3 hold' are not known but may well include the downregulation of *Ednrb* and the spatial and temporal regulation of *Edn3* expression within the gut. Therefore, by regulating the expression of receptors for multiple signalling pathways, *Sox10* is uniquely capable of coordinating the transition from a multilineage progenitor to a differentiated cell type in the specific microenvironment of the developing mammalian ENS.

The smaller pool of *Sox10*-expressing ENS progenitors observed in $Edn3^{ls}$ homozygote embryos results, at least partly, from reduced proliferation of these cells. Although EDN3 is a mitogenic factor for avian neural crest (Lahav, 2005; Lahav et al., 1998; Lahav et al., 1996), no direct proliferative effect has so far been described for mammalian enteric neural crest cells or EPCs. Therefore, EDN3 might influence the proliferation of endogenous ENS progenitors indirectly (Barlow et al., 2003), perhaps by maintaining their undifferentiated state. Contrary to our findings, other investigators have reported that *Ednrb* mutations in rats have no effect on the proliferation of enteric neural crest cells during embryogenesis (Kruger et al., 2003). Moreover, these authors presented evidence that EDN3 promotes the differentiation of cultured NCSCs to myofibroblasts and that absence of EDNRB signalling in vivo has no effect on neuronal differentiation. The reasons for the discrepancy between this report and our studies are currently unclear. It is possible that species specific differences could determine the extent to which endothelin signalling can influence the proliferative and differentiation responses of neural crest cells. Alternatively, these discrepancies could reflect potential differences in the cell populations analysed by the two laboratories. Further experimentation will be necessary to explore these issues.

One of the most striking findings of our work is that the effect of the $Edn3^{ls}$ mutation on endogenous enteric neural crest cells varies along the length of the embryonic gut. Thus, cells located at the migratory front are more dependent on endothelin signalling for their proliferation relative to other more posterior cell groups. Moreover, $Edn3^{ls/ls}$ embryos show increased differentiation at the front of migration, although overall the differentiation of enteric neural crest cells is reduced. The increased differentiation and reduced proliferation of neural crest cells at the migratory front are likely to reflect primary effects of EDN3 on ENS progenitors, consistent with the distribution of Edn3 mRNA in vivo and the observed effects of EDN3 on clonogenic EPC cultures. By contrast, the effects of the $Edn3^{ls}$ mutation on more posterior groups of enteric neural crest cells might reflect secondary effects of the EDN3 reduction. For example, the overall reduction in the fraction of $TuJ1^{+}$ cells in the gut of mutant embryos could be a consequence of a reduced total number of neuroectodermal cells within the mutant gut (itself the result of reduced proliferation and increased differentiation of the early neural crest invading the gut), which could prevent the necessary cell-cell interactions to drive cell differentiation. Irrespective of the specific mechanisms underlying this effect, our findings reveal that the effect of mutations in *Edn3* are spatially and temporally regulated, consistent with the implication of this signalling pathway in the integration, in time and in space, of cellular activities necessary for normal ENS neurogenesis.

CEE is a key ingredient of the culture medium used for the propagation of multipotential self-renewing progenitors of the PNS, including NCSCs and EPCs (Bondurand et al., 2003; Morrison et al., 1999; Stemple and Anderson, 1992). As CEE is an unfractionated homogenate of chick embryos, its crucial ingredient(s) are currently unknown. Here, we demonstrate that EDN3 signalling is necessary and sufficient to support the formation of NLBs and the recovery of EPCs in dissociated embryonic gut cultures in the absence of CEE, suggesting that EDN3 is an active ingredient of CEE and mediates its effects on PNS progenitors. Although the mechanisms by which endothelin signalling preserves the undifferentiated state of EPCs are currently unclear, our findings could have profound effects on stem cell biology as they suggest that EDN3 is uniquely capable of converting an asymmetric pattern of cell divisions of ENS progenitors (EPCs), which in standard media generate colonies containing a mixture of multilineage progenitors and differentiated progeny, to a symmetrical one that gives rise to colonies composed almost exclusively of multilineage progenitors. A similar potential has been assigned recently to FGF2 and EGF, which, in combination, suppress the differentiation of neural stem (NS) cell lines and support their continuous symmetrical self-renewal divisions (Conti et al., 2005). It is currently unknown whether EDN3, alone or in combination with other factors, can maintain the long-term self-renewal of undifferentiated neural crest cells in culture. Nevertheless, our findings suggest that EDN3 could be a key ingredient of fully defined culture media that would allow the expansion of a homogeneous population of ENS stem cells that, upon removal of this factor, would be able to differentiate into enteric neurons and glia. Such a cell population could be used for cell transplantation experiments aimed at restoring the peristaltic activity of aganglionic gut segments.

Note added in proof

Just before our paper was accepted another publication appeared that, in general, is in agreement with the conclusions of our work (Nagy and Goldstein, 2006).

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