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# Sonic hedgehog regulates the proliferation, differentiation, and migration of enteric neural crest cells in gut

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Enteric neural crest cells (NCCs) migrate and colonize the entire gut and proliferate and differentiate into neurons and glia of the enteric nervous system in vertebrate embryos. We have investigated the mitogenic and morphogenic functions of Sonic hedgehog (Shh) on enteric NCCs in cell and organ culture. Enteric NCCs expressed Shh receptor Patched and transcripts encoding the Shh signal transducer (Gli1). Shh promoted the proliferation and inhibited the differentiation of NCCs. The pro-neurogenic effect of glial cell line-derived neurotrophic factor (GDNF) on

NCCs was abolished by Shh. In gut explants, NCCs migrated from the explants onto the adjacent substratum if GDNF was added, whereas addition of Shh abolished this migration. Neuronal differentiation and coalescence of neural crest-derived cells into myenteric plexuses in explants was repressed by the addition of Shh. Our data suggest that Shh controls the proliferation and differentiation of NCCs and modulates the responsiveness of NCCs toward GDNF inductions.

## Introduction

In vertebrate embryos, enteric neural crest cells (NCCs) from the vagal level of the neural tube provide the enteric nervous system (ENS). These cells enter the foregut and then colonize the entire gut in a rostro-caudal wave. Besides this primary wave of rostro-caudal migration, a secondary migration wave of NCCs also takes place across the gut radius (for review see Newgreen and Young, 2002). This secondary migration is implicated in the formation of the two neural plexuses that make up the ENS: myenteric plexus between the longitudinal and circular muscle, and submucosal plexus on the luminal side of the circular muscle. The migration, proliferation, and differentiation of NCCs are dependent on reciprocal interactions between NCCs, the gut mesenchyme, and the gut endoderm. Gut mesenchyme-derived factors and their receptors on NCCs, namely glial cell line-derived neurotro-

phic factor (GDNF) and its receptors, rearrangement during transformation (RET) and GFRA1, and endothelin 3 (EDN3) and the endothelin receptor-B (EDNRB), are crucial for the rostro-caudal migration of NCCs (for review see Chakravarti and Lyonnet, 2001; Barlow et al., 2003; Kruger et al., 2003). Sonic hedgehog (Shh) and Netrins have been suggested to regulate the secondary migration of NCCs (Sukegawa et al., 2000; Jiang et al., 2003).

Shh belongs to the Hedgehog family of signaling molecules that were identified by their homology to the *Drosophila melanogaster* segment polarity gene hedgehog. Shh controls cellular differentiation and proliferation in a variety of tissues (for review see Ingham and McMahon, 2001; McMahon et al., 2003). As the gut tube is formed, Shh is expressed throughout the rostro-caudal extent of the gut endoderm in all vertebrates examined (Echelard et al., 1993; Krauss et al., 1993; Bitgood and McMahon, 1995; Marigo et al., 1995; Roberts et al., 1995; Stolow and Shi, 1995). Autoproteolytic

M. Fu and V.C.H. Lui contributed equally to this paper.

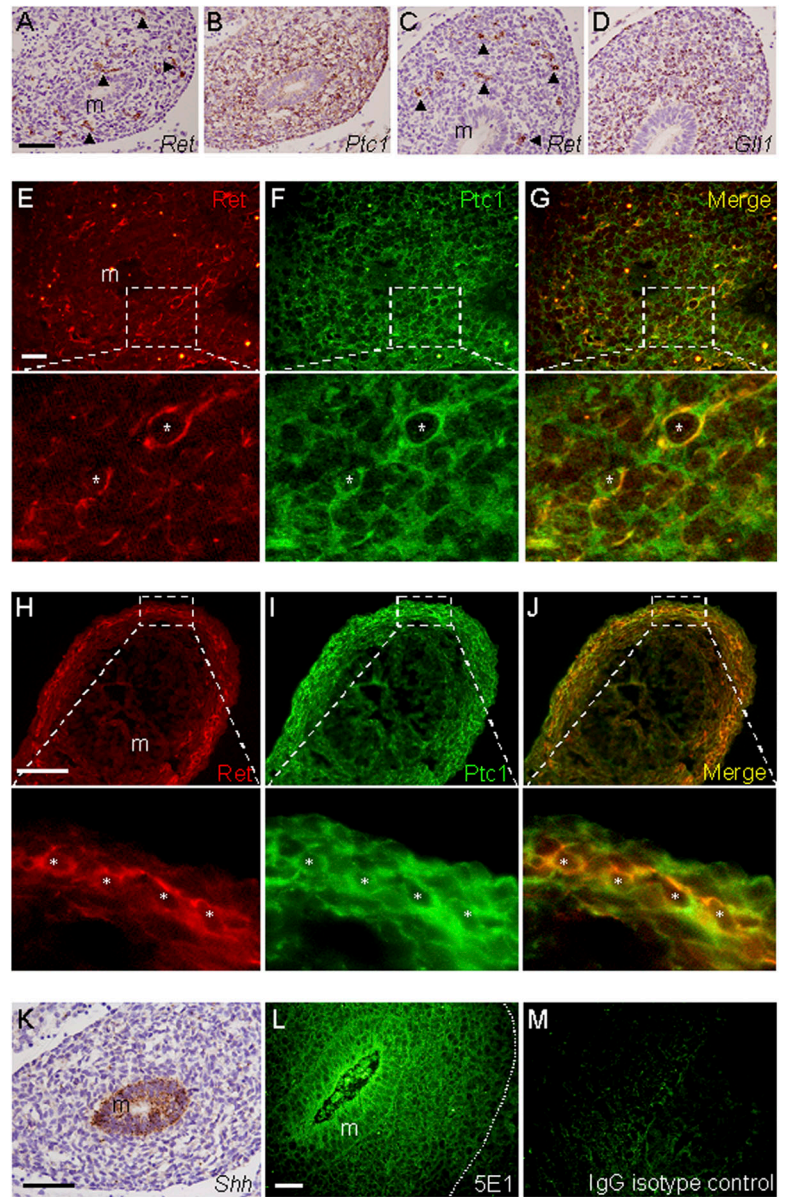
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Key words: Sonic hedgehog; enteric nervous system; neural plexuses; neurogenesis; glial cell-line derived neurotrophic factor

Abbreviations used in this paper: EDN3; endothelin 3; EDNRB; endothelin receptor-B; ENS, enteric nervous system; GDNF, glial cell line-derived neurotrophic factor; NCAM, neural cell adhesion molecule; NCC, neural crest cell; Ptc1, Patched; Ret, rearrangement during transformation; Shh, Sonic hedgehog; SMA, smooth muscle  $\alpha$ -actin; TH, tyrosine hydroxylase; TUJ1, neuron-specific  $\beta$ -tubulin.

**Figure 1. Expression of *Ptc1* and *Gli1* in mouse embryonic guts was analyzed by in situ hybridization.** NCCs that expressed *Ret* were localized within the E11.5 gut mesenchyme (A and C, arrowheads). Transcripts of *Ptc1* (B) and *Gli1* (D) were localized within the mesenchyme. Using anti-*Ret* and -*Ptc1* antibodies, *Ptc1* protein was detected at the NCCs (asterisks) and the mesenchyme of the E11.5 gut (E–G) and E13.5 gut (H–J). Transcripts of *Shh* were restricted to the mucosa (K). Immunofluorescence using antibody 5E1 localized *Shh* protein at the mucosa and mesenchyme of the E11.5 gut (L). Staining with IgG isotype control showed a weak nonspecific signal (M). Dashed lines in L mark the gut border. m, mucosa. Photos A–G and K–M were taken at the same magnification. Photos H–J were taken at the same magnification. Bars: (A–G and K–M) 100  $\mu$ m; (H–J) 50  $\mu$ m.



cleavage of Shh generates the active NH<sub>2</sub>-terminal peptide (N-Shh) that regulates proliferation and differentiation of different cell types in the gut mesenchyme and in neural crest-derived cells (Bitgood and McMahon, 1995; Marigo et al., 1995; Sukegawa et al., 2000). Colonization of the gut by NCCs is completed in *Shh*<sup>-/-</sup> mice. However, abnormal neural plexus formation and placement in *Shh*<sup>-/-</sup> mice suggests that Shh is involved in the spatial development of neural plexuses (Ramalho-Santos et al., 2000). Shh promotes the proliferation of mouse cerebellar granule neuron precursors directly by the induction of Nmyc and G<sub>1</sub> cyclins (Kenney and Rowitch, 2000; Kenney et al., 2003). Shh promotes the survival and proliferation of hindbrain NCCs, the motoneurons of neural tube, and the neuron progenitors of the telencephalon (Rowitch et al., 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999; Britto et al., 2000, 2002; Machold et al., 2003). However, a direct regulatory function of Shh on enteric NCCs has yet to be shown. It has been suggested that Shh controls ENS development indirectly by

the induction and secretion of mesenchyme-derived factors, which in turn regulates NCC development (Roberts et al., 1995; Sukegawa et al., 2000).

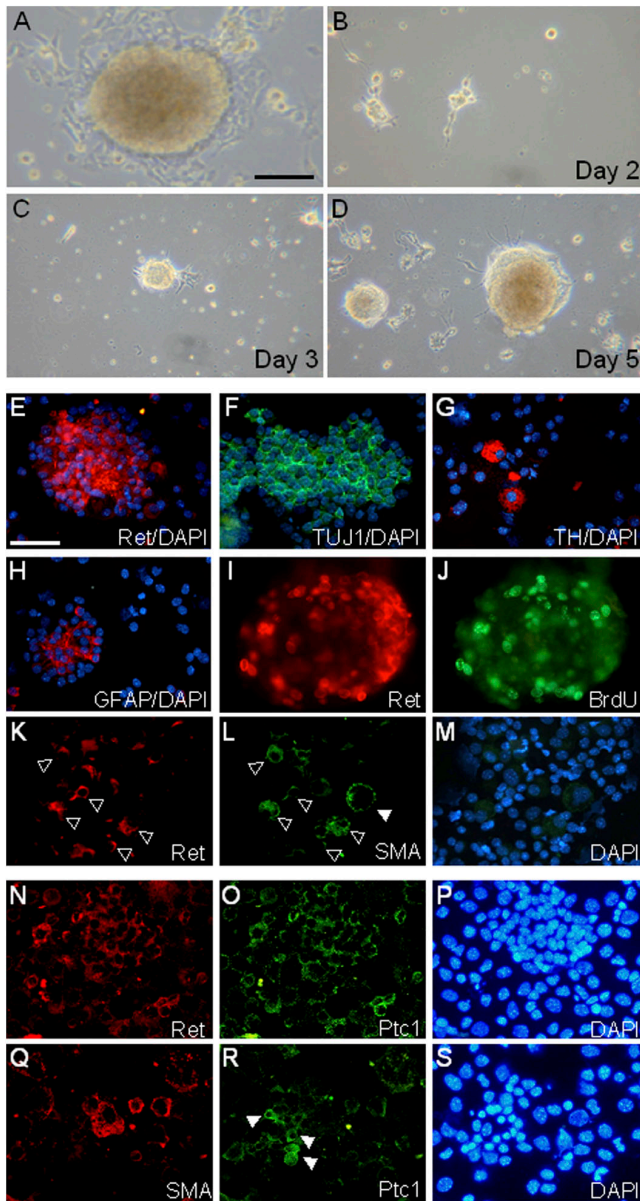
In this work, we investigated the regulation of Shh in the proliferation, differentiation, and migration of enteric NCCs using neurosphere culture, explants, and dissociated gut culture of mouse embryonic gut. We present evidence indicating that Shh may regulate the development of neural plexuses by the modulation of the responsiveness of NCCs toward GDNF inductions and/or the regulation of the proliferation and differentiation of NCCs in the gut.

## Results

### Enteric NCCs express Shh receptor and signal transducer

Spatio-temporal expression of *Ptc1* and *Gli1* in mouse embryonic guts was analyzed by in situ hybridization and immunohistochemistry. NCCs that expressed *Ret* were localized within the E11.5 mouse gut mesenchyme (Fig. 1, A and





**Figure 2. Neurospheres contain self-renewable and multipotent NCCs.** (A) Primary neurospheres were formed at day eight. After replating at a clonal density, small clusters were seen at day two (B), which domed (C) and developed into neurospheres at day five (D). Immunofluorescence analyses of cytospin preparations of neurospheres were performed to localize Ret<sup>+</sup> cells (E and K), TUJ1<sup>+</sup> neuron progenitors/neurons (F), TH<sup>+</sup> neurons (G), GFAP<sup>+</sup> glia (H), and SMA<sup>+</sup> myofibroblasts (L). Neurosphere preparation was stained for Ret (K), SMA (L), and DAPI (M). Whole-mount immunofluorescence and BrdU incorporation assays of neurosphere showed that Ret<sup>+</sup> cells incorporated BrdU (I and J). Immunocolocalization analyses indicated that Ptc1 (O) immunoreactivity overlapped with that of Ret (N). In contrast, cells showing intense Ptc1 staining (R, closed arrowheads) were SMA<sup>-</sup> (compare Q with R). Neurosphere preparation was stained either for Ret (N), Ptc1 (O), and DAPI (P) or for SMA (Q), Ptc1 (R), and DAPI (S). Photos A–D were taken at the same magnifications. Photos E–S were taken at the same magnifications. Bars: (A–D) 50  $\mu$ m; (E–S) 25  $\mu$ m.

C). Transcripts of *Ptc1* and *Gli1* were localized within the mesenchyme (Fig. 1, B and D). Patched (Ptc1) immunoreactivity was localized at the E11.5 gut mesenchyme and Ret<sup>+</sup> NCCs (Fig. 1, E–G). At E13.5, Ptc1 was detected at the mesenchyme and at the presumptive myenteric region underneath the serosa where Ret<sup>+</sup> NCCs were found (Fig. 1, H–J). Transcripts of *Shh* were restricted to the mucosa of the E11.5 gut (Fig. 1 K). However, Shh protein was detected at the mucosa and mesenchyme by immunofluorescence (Fig. 1 L). The signal was strong at the mucosa, whereas the signal at the mesenchyme declined the further it was away from the region adjacent to the mucosa. These findings indicated that Shh was secreted from the mucosa into the mesenchyme forming a concentration gradient across the gut radius. Staining with mouse IgG isotype control antibody gave a weak nonspecific signal (Fig. 1 M).

### Neurospheres contain self-renewable multipotent NCCs

To prepare the neurosphere of enteric NCCs, dissociated E11.5 mouse guts were cultured on coated dishes. Mesenchyme cells grew as a monolayer and NCCs formed clusters. Mesenchyme cells grow poorly, and were removed in each replating. Very few mesenchyme cells remained in culture after five passages (i.e.,  $\sim$ 8–10 d from day one of culture). Clusters of cells were first seen at 6 d. These clusters formed domes and later developed into primary neurospheres at day eight (Fig. 2 A). If neurospheres were replated at clonal density, small clusters could be observed at day two growing into secondary neurospheres at day five (Fig. 2, B–D). By replating at a clonal density, neurosphere cells were capable of being maintained in culture for longer than 60 d, which was equivalent to more than 10 passages; this indicated self-renewal of neurosphere cells.

To determine cellular compositions of neurospheres, cytospin preparations of day seven neurospheres were stained for Ret (marker for NCCs, neuron progenitors/neurons), neuron-specific  $\beta$ -tubulin (TUJ1; marker for neuron progenitors/neurons), tyrosine hydroxylase (TH; marker for catecholamine-synthesizing neurons), GFAP (glial marker), and smooth muscle  $\alpha$ -actin (SMA; myofibroblast marker). Ret<sup>+</sup> (Fig. 2 E) cells, TUJ1<sup>+</sup> (Fig. 2 F) cells, TH<sup>+</sup> neurons (Fig. 2 G), and GFAP<sup>+</sup> glia (Fig. 2 H) were detected in the culture. SMA<sup>+</sup> cells were mostly immunoreactive for Ret (Fig. 2, open arrowheads), but SMA<sup>+</sup>/Ret<sup>-</sup> cells (Fig. 2,

**Table 1. Cellular compositions of day seven neurospheres**

Antibody staining	Percentage of neurosphere cells (n = 6)
Ret <sup>+</sup>	53 $\pm$ 12
TUJ1 <sup>+</sup>	40 $\pm$ 8
TH <sup>+</sup>	7.5 $\pm$ 2
GFAP <sup>+</sup>	16.1 $\pm$ 4.4
SMA <sup>+</sup> /Ret <sup>+</sup>	10 $\pm$ 2
SMA <sup>+</sup> /Ret <sup>-</sup>	2.4 $\pm$ 0.5
Ret <sup>-</sup>	47 $\pm$ 12

n equals the number of neurosphere cultures analyzed, and at least three randomly chosen fields from each culture were counted.

closed arrowhead) could also be detected (compare Fig. 2, K and L). No overlapping of TUJ1 and GFAP immunoreactivity was observed. The percentage of neurosphere cells expressing these markers was quantitated as shown in Table I. Ret<sup>-</sup> cells may consist of glia, Ret<sup>-</sup>/SMA<sup>+</sup> myofibroblasts, undifferentiated mesenchyme cells, and/or endoderm cells. Half of the Ret<sup>+</sup> cells ( $55 \pm 2\%$ ;  $n = 6$ ) incorporated BrdU, indicating that some Ret<sup>+</sup> cells were proliferative (Fig. 2, I and J). These findings indicated that neurospheres contained enteric NCCs and that these NCCs self-renewed and differentiated into cells of multi-lineages.

To determine if NCCs and their derivatives expressed the Shh receptor Ptc1, immunofluorescence staining using antibodies specific for Ret, Ptc1, and SMA on cytospin preparations of day 12 neurospheres was performed. Immunocolocalization study showed that Ret immunoreactivity largely overlapped with that of Ptc1 (compare Fig. 2, N–P). In contrast, cells showing intense Ptc1 staining (Fig. 2 R, arrowheads) were negative for SMA (compare Fig. 2, Q with R). These findings suggested that NCCs, neuron progenitors, and neurons expressed Ptc1.

### Shh promotes proliferation of neurosphere cells

To study the effect of Shh on the growth and differentiation of enteric NCCs, we cultured neurospheres in the presence or absence of Shh and analyzed the cultures by immuno-

Table II. Cellular compositions of neurospheres in the presence or absence of Shh

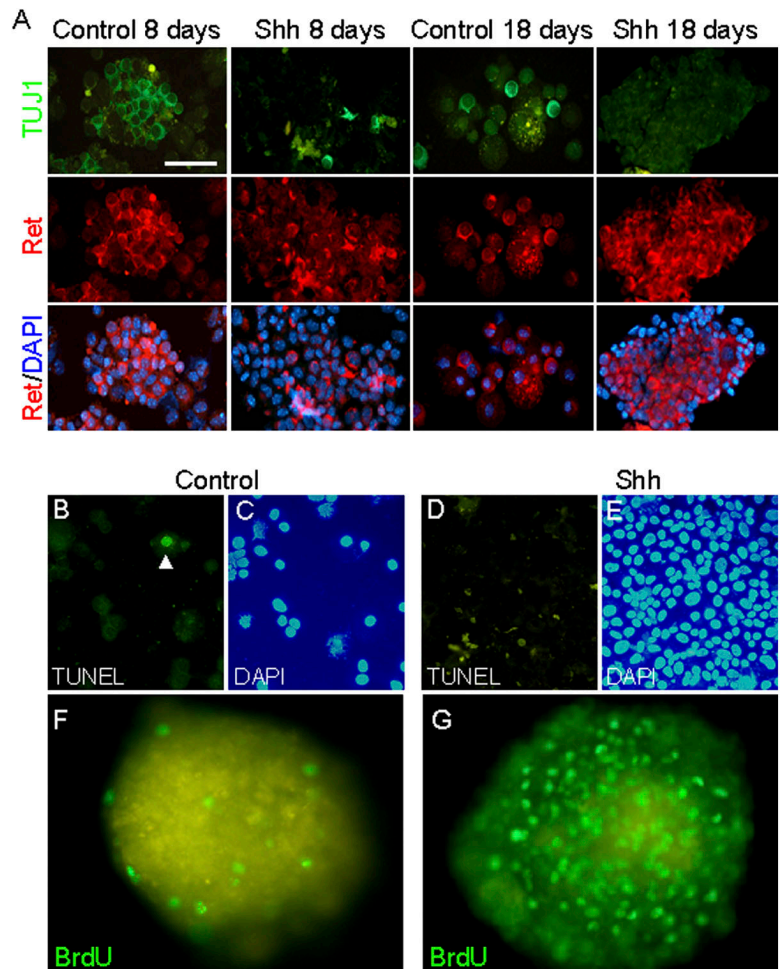
	Neurosphere cells expressing different markers			
	8 d		18 d	
	Control	Shh	Control	Shh
Ret <sup>+</sup>	54.2 ± 3.8	52.5 ± 6.9 (P = 0.8)	13.3 ± 1.8	55 ± 23.2 (P = 0.04)
Ret+/TUJ1+	46 ± 7.8	18.2 ± 5.9 (P = 0.05)	1 ± 1	0.3 ± 0.3 (P = 0.1)
Ret+/TUJ1-	8.2 ± 4.2	35.3 ± 5.2 (P = 0.004)	12.3 ± 2.4	54.6 ± 23.3 (P = 0.05)
Total cell count	107 ± 12.6	112 ± 7.4 (P = 0.99)	46.8 ± 5.9	61.7 ± 24.8 (P = 0.9)

Cell counting was performed on at least three randomly chosen fields from each culture. Data are mean ± SEM. Six neurosphere cultures were analyzed for each treatment. *t* test was performed.

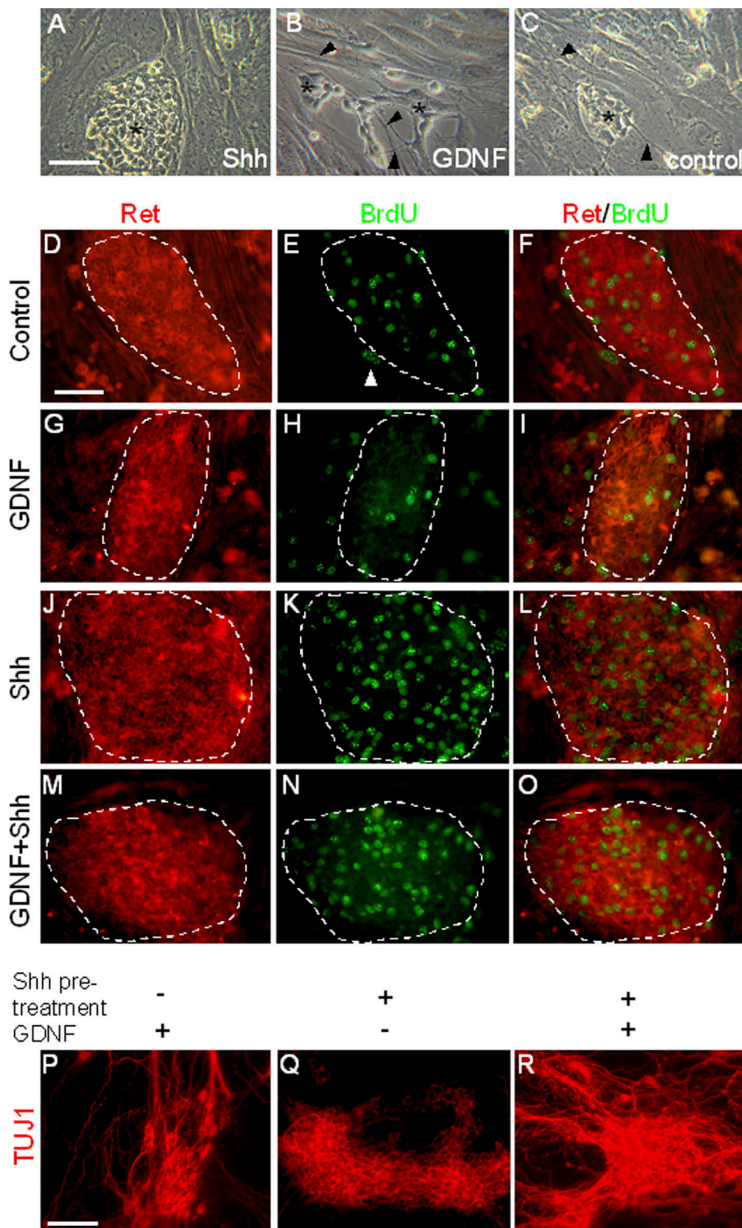
staining using anti-Ret and anti-TUJ1 antibodies (Fig. 3 A). NCCs maintained Ret expression and expressed neuron markers such as TUJ1, as they were committed to neuronal lineage. In contrast, NCCs switched off expressing Ret once they were committed to glial differentiation. We described NCCs as Ret<sup>+</sup>/TUJ1<sup>-</sup> and neuron progenitors/neurons as Ret<sup>+</sup>/TUJ1<sup>+</sup> cells in our cultures. Numbers of cells expressing Ret with or without coexpression of TUJ1 in control and Shh-treated neurospheres were counted (Table II).

Figure 3. Shh promotes the proliferation of NCCs.

(A) Cytospin preparations of neurospheres that had been cultured without (control) or with (Shh) Shh were stained for TUJ1 (green) or Ret (red). TUNEL was performed to localize apoptotic cells (green; arrowhead) in neurospheres cultured without (control; B and C) or with Shh (D and E). Control neurosphere (10 d culture) contained very few BrdU<sup>+</sup> cells (F). In contrast, neurospheres cultured with Shh for 10 d contained numerous BrdU<sup>+</sup> cells (G). Bar, 50 μm.







**Figure 4. Shh suppresses the differentiation of NCCs.**

NCC clusters were seen after 18 h of incubation, and clusters increased in size after two more days in Shh culture (A, asterisk). NCC clusters (asterisks) could also be found in GDNF culture (B) and in control culture (C; plain medium) after 40 h of incubation. Neurite projections (arrowheads) were obvious in GDNF culture (B) and in control culture (C). (D–O) NCC clusters were localized by Ret staining (red; marked with dashed lines), and proliferative cells in the clusters were detected with anti-BrdU antibody (green). BrdU+ cells were localized within the clusters (E, H, K, and N), and very few mesenchyme cells were found to be BrdU+ (E, arrowhead). NCC clusters pretreated with Shh (Q and R) or without Shh (P) were incubated with new media with (P and R) or without (Q) GDNF. Cultures were stained for TUJ1 (P–R, red). All photos were taken at the same magnification. Bars, 100  $\mu$ m.

NCCs (Ret+/TUJ1-) were more abundant in Shh culture than in the control medium. Conversely, neuron progenitors/neurons (Ret+/TUJ1+) were less abundant in culture with Shh than in the control medium. TUNEL staining on cytospin preparations of day 10 neurospheres revealed that apoptotic cells were rarely seen in cultures with or without Shh (Fig. 3, B–E). Shh-treated neurospheres (Fig. 3 G) contained 3.5-fold more BrdU+ cells than that in control (without Shh; Fig. 3 F; BrdU+ cells in Shh treatment vs. control:  $74 \pm 2.7$  vs.  $20 \pm 1.4$ ;  $P = 0.0001$ ;  $t$  test;  $n = 10$ ).

#### Comparison of effects of Shh and GDNF on dissociated gut cultures

NCC clusters were seen in Shh culture after 18 h of incubation, and the clusters increased in size after two more days (Fig. 4 A). NCC clusters were detected only after 3–4 d of incubation with GDNF (Fig. 4 B) and were generally smaller than those formed in the presence of Shh (compare

Fig. 4 A and Fig. 4 B). Only few and relatively small clusters were observed in the culture with plain medium (Fig. 4 C, control). Cells of clusters in different treatments displayed variable morphologies. Neuronal processes (Fig. 4, arrowheads) were scarce in Shh culture, but were very prominent in the cultures with GDNF and in control (compare Fig. 4, A–C). Dissociated gut cells had been filtered through cell strainers to remove cell clumps before plating, so NCC cluster formation in culture was not the result of insufficient dissociation of gut cells. Bigger size of clusters in culture could reflect a higher proliferation of NCCs.

Proliferation of NCCs was assayed by BrdU incorporation. NCC clusters, which were marked by Ret, contained BrdU+ proliferating NCCs (Fig. 4, D–O). Proliferating NCCs were more abundant in clusters formed in the presence of Shh (Fig. 4, J–L) than in its absence (Fig. 4, D–F) and in the presence of GDNF (Fig. 4, G–I). BrdU+ NCCs were more abundant in the culture with GDNF plus Shh (Fig. 4, M–O) than in

Table III. **Shh promotes NCC proliferation in dissociated gut**

Treatment (8 d)	Control	Shh
Percentage of Ret+ cells	66.9 ± 9.4	78.8 ± 4.9
Percentage of Ret+ cells that was BrdU+	21.6 ± 5.4	70.4 ± 6.5 <sup>a</sup>
Percentage of TUJ1+ cells	19.4 ± 8.3	23.1 ± 5.2
Percentage of TUJ1+ cells that was BrdU+	65 ± 4.2	54.3 ± 3

t test was performed. Data are mean ± SEM. Six cultures were analyzed for each treatment. Cell counting was performed on at least six randomly chosen fields from each culture.

<sup>a</sup>Only the Ret+ cells between control and Shh treatment showed significant difference, P = 0.00001.

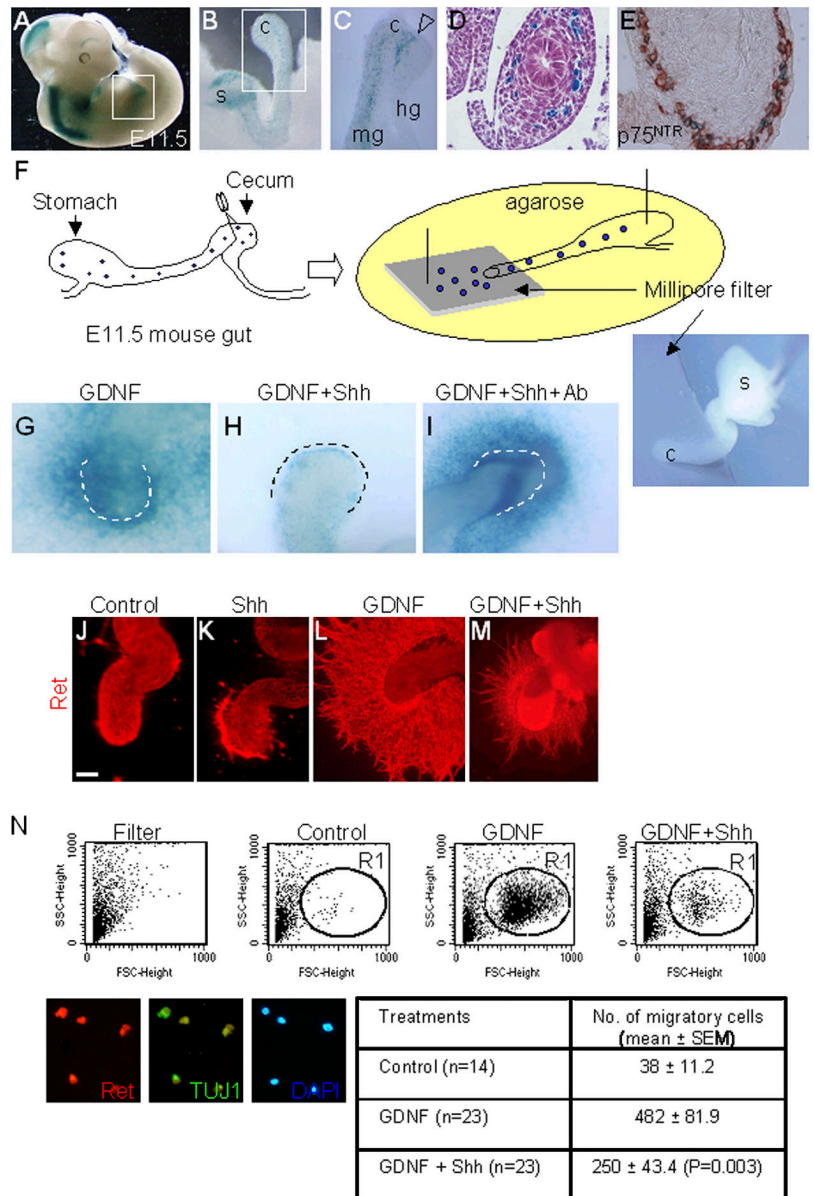
that with GDNF alone (Fig. 4, G–I). Percentages of Ret+ and TUJ1+ cells were not statistically different between cultures with or without Shh (Table III). In contrast, Shh culture contained a significantly higher proportion of Ret+ cells that were BrdU+ than in culture with plain medium (control).

These findings suggested that Shh promoted the proliferation of NCCs in dissociated gut culture.

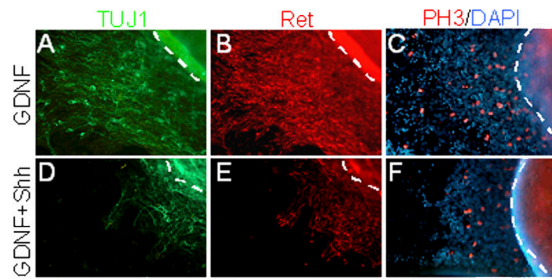
To investigate if Shh-treated enteric NCCs retained neurogenic potential and differentiated into neurons upon GDNF induction, we precultured NCC clusters with Shh for 2 d, then added fresh medium with or without GDNF and cultured for another 2 d. Clusters were generally bigger in Shh pretreated cultures (Fig. 4, Q and R) than those without pretreatment (Fig. 4 P). NCC clusters with Shh pretreatment differentiated into TUJ1+ neurons with long neuronal processes after GDNF induction (Fig. 4 R). Neuronal differentiation of Shh pretreated NCC clusters was also observed without GDNF induction (Fig. 4 Q), but long neuronal processes were undetected. NCC clusters that had been cultured with Shh for 30 d also differentiated into neurons upon GDNF induction (not depicted). These findings indicated that Shh pretreated NCCs were responsive to GDNF and differentiated into neurons.

Figure 5. **Shh restricts the migration of NCCs.**

X-gal/IPTG staining of E11.5 *b3-IIIa-LacZ* transgenic embryo indicated β-galactosidase activities (blue) were detected at the anterior spinal cord, hindbrain, and intestine (A and B, insets) reaching the cecum (C, arrowhead). Sections of the X-gal/IPTG-stained E11.5 foregut revealed β-galactosidase-expressing cells in the mesenchyme (D) and colocalized with p75<sup>NTR</sup> immunostaining signals (E). Gut explants of E11.5 transgenic mouse embryos were established as shown (F). After 3 d of incubation with GDNF (G), GDNF + Shh (H), and GDNF + Shh + Shh neutralizing antibody (I), explants together with the filter paper were stained with X-gal/IPTG. Explants of E11.5 nontransgenic mouse guts were incubated in different conditions for 3 d and stained for Ret (J–M, red). Numbers of migratory cells on filters in different treatments were quantified by flow cytometry (N). Cells selected for counting are highlighted with circle (R1). The cells that were collected from filters for counting expressed Ret and TUJ1. Cell counts in each treatment were determined and tabulated as shown (n, number of explants in each treatment). s, stomach; c, cecum; mg, midgut; hg, hindgut. Photos J–M were taken at the same magnification. Bar, 100 μm.







Treatment	$\frac{\text{PH3+ cells}}{\text{total cells}} \times 100\%$
GDNF (n=9)	$6.5 \pm 3$
GDNF + Shh (n=9)	$7.8 \pm 4^*$

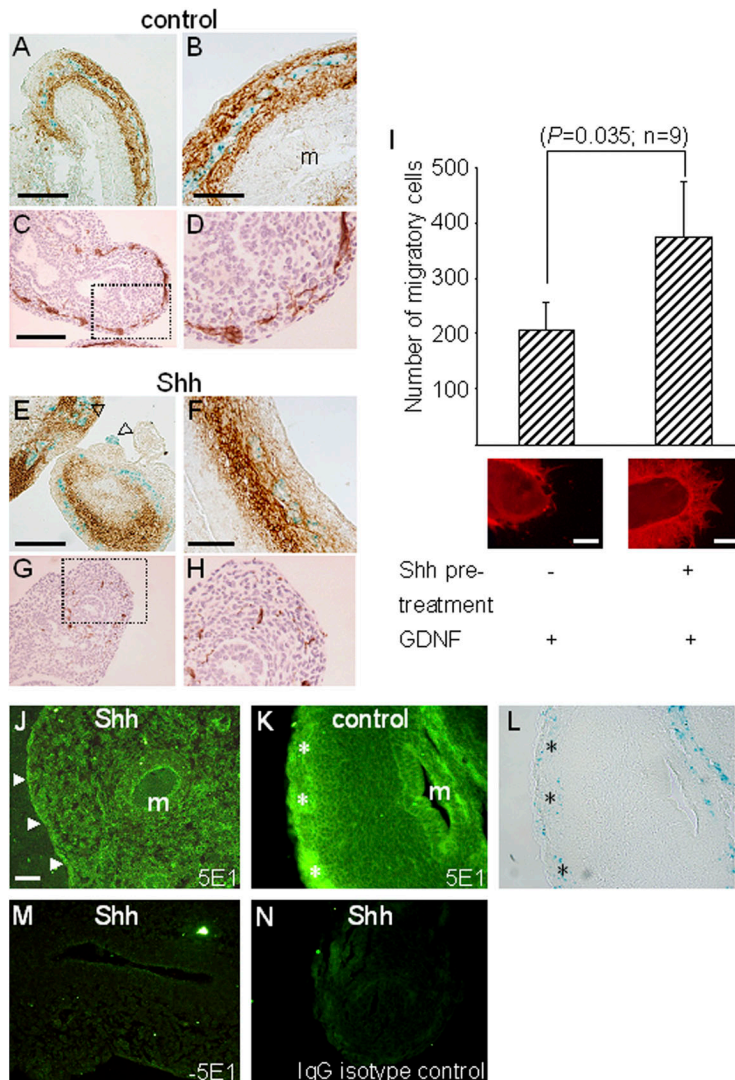
\*  $P > 0.05$

**Figure 6. Shh restricts the migration of NCCs.** Migratory cells on the filter from explants cultured for 3 d in the presence of GDNF (A–C) or GDNF + Shh (D–F) were stained for TUJ1 (A and D, green), Ret (B and E, red), or PH3 (C and F, red). PH3+ cells and total number of cells on the membrane in each treatment were counted (n, number of explants in each treatment); and percentage of PH3+ cells was calculated and tabulated as shown.

### Shh restricts the migration of enteric NCCs

To investigate the effect of Shh on NCC migration, we established ex vivo gut explant cultures of *b3-IIIa-LacZ* transgenic and nontransgenic mice. A 500-base cis-acting enhancer of mouse *Hoxb3* gene directs the  $\beta$ -galactosidase expression to the hindbrain at rhombomeres 6–8, the anterior spinal cord, and the associated NCCs in *b3-IIIa-LacZ* transgenic mice (Yau et al., 2002). Migrating NCCs and neural plexus of transgenic gut were stained blue by X-gal/IPTG, indicative of  $\beta$ -galactosidase activity, and expressed NCC marker p75<sup>NTR</sup> (Fig. 5, A–E). In the presence of GDNF,  $\beta$ -galactosidase-expressing NCCs migrated onto the membrane (Fig. 5 G). Addition of Shh reduced NCC migration (Fig. 5 H), and Shh inhibition was partially abolished by Shh-neutralizing antibody (Fig. 5 I). Inhibition of Shh on NCC migration was also observed in nontransgenic explants (compare Fig. 5, J–M).

The migratory cells that were collected from the membrane for counting expressed Ret and TUJ1, confirming that these cells were derivatives of enteric NCCs (Fig. 5 N). Few NCCs migrated out of the explants in the absence of GDNF. Addition of Shh reduced the number of migratory NCCs by half, as compared with those treated with GDNF



### Figure 7. Shh represses the formation of neural plexuses.

Sections of X-gal/IPTG-stained *b3-IIIa-LacZ* explants that had been treated with (Shh) or without (control) Shh for 2 d were stained for SMA (A, B, E, and F; brown), and NCCs were localized by X-gal/IPTG staining (blue). Sections of Shh-treated or control nontransgenic explants were stained for TUJ1 (C, D, G, and H; brown). Small clumps of NCCs (stained blue) were localized to the explant surface (E, open arrowheads). The boxed regions were magnified as shown on the right. Gut explants were precultured for 2 d with or without Shh, and the medium was replaced by medium with or without GDNF and incubated for two more days. The cultures were stained for TUJ1 (I, red). Numbers of migratory cells on filters in different treatments were quantified by flow cytometry. Average cell count  $\pm$  SEM were determined and shown as a histogram (n, number of explants in each treatment). Sections of Shh-treated transgenic explant were stained with anti-Shh antibody (J), blocking solution (M), or isotype control antibody (N). Immunofluorescence (K) and phase-contrast (L) of the control explant that was stained with anti-Shh antibody are shown. (J) Serosa (arrowheads) of Shh-treated explants displayed strong immunoreactivity for Shh. The  $\beta$ -galactosidase-expressing NCCs (L, asterisks) at the presumptive myenteric region of control explants showed strong auto-fluorescence (K, asterisks). Photos C, D, G, and H were taken at the same magnification. Photos J–N were taken at the same magnification. m, mucosa. Bars: (A and E and J–N) 50  $\mu\text{m}$ ; (B and F) 25  $\mu\text{m}$ ; (C, D, and G–I) 100  $\mu\text{m}$ .



Table IV. **Shh promotes NCC proliferation in gut**

Treatment (3 d)	Cell counts in cell clumps		
	Ret+	Ret+/BrdU+	Total cells
Control	21.4 ± 9.5 (82.8 ± 5%)	4.8 ± 1.9 (22.6 ± 4%)	24.2 ± 9.7
Shh	66.6 ± 15.8 (92.3 ± 2%) (P = 0.5 <sup>a</sup> )	34 ± 9.7 (48.4 ± 3%) (P = 0.008 <sup>a</sup> )	72.2 ± 17.2
GDNF	68.4 ± 6.9 (87.1 ± 2%) (P = 1 <sup>a</sup> ; 0.6 <sup>b</sup> )	12 ± 2.7 (17.2 ± 2%) (P = 0.8 <sup>a</sup> ; 0.008 <sup>b</sup> )	82.4 ± 6.8
Shh + GDNF	79 ± 6.2 (87.4 ± 4.5%) (P = 0.9 <sup>a</sup> ; 0.9 <sup>b</sup> ; 0.9 <sup>c</sup> )	33.4 ± 4.6 (43.1 ± 6.6%) (P = 0.06 <sup>a</sup> ; 0.7 <sup>b</sup> ; 0.01 <sup>c</sup> )	90.8 ± 6.6

Data are mean ± SEM,  $n = 9$ . Cells were counted in nine explants from three sets of experiments; and three views were randomly chosen for counting. Percentage of Ret+ and Ret+/BrdU+ cells with respect to total cells in cell clumps were calculated from each experiment, and then the overall mean percentage ± SEM was calculated and shown in parenthesis. ANOVA with Tukey post-hoc corrections was performed for multiple comparisons. P values are shown.

<sup>a</sup>Comparison with control.

<sup>b</sup>Comparison with Shh culture.

<sup>c</sup>Comparison with GDNF culture.

alone (Fig. 5 N;  $P = 0.003$ ;  $t$  test). NCCs on the membrane had differentiated into neurons as they expressed TUJ1 (Fig. 6, A and D). However, some of these TUJ1+ cells were still proliferating as revealed by PH3 immunoreactivity (Fig. 6, A–F). The proportion of PH3+ cells on the membrane was similar between the cultures with or without Shh, indicating that the proliferation of cells on the membrane was comparable in these cultures ( $P > 0.05$ ;  $t$  test). This finding suggested that the variable numbers of cells on the membrane in various treatments was not the consequence of different proliferation of migratory cells on the membrane. In other words, the numbers of cells on the membrane could reflect the number of migratory NCCs from the explants in response to different factors.

### Shh disrupts the development of enteric neural plexus

Sections of Shh-treated explants and control explants (without Shh) of *b3-IIIa-LacZ* mice were stained for SMA and TUJ1 to study the concentric organization of gut explant and neuronal differentiation of NCCs. Two distinct layers of SMA+ myofibroblasts were detected in the mesenchyme of control explant suggesting that the circular and longitudinal muscle were developing properly (Fig. 7, A and B). NCCs (Fig. 7, A and B, blue) and TUJ1+ neurons (Fig. 7, C and D) coalesced at the presumptive myenteric region between the developing muscle layers. In contrast, well-defined layers of SMA+ myofibroblasts were not developed in Shh-treated explants, and the presumptive myenteric region was indiscernible (Fig. 7, E and F). In Shh-treated explants, NCCs randomly aggregated into clumps (Fig. 7 E, arrowheads), and TUJ1+ neurons were sparse and randomly distributed (Fig. 7, G and H). These findings indicated that neuronal differentiation of NCCs was repressed by Shh, and that neuronal cells failed to coalesce at the presumptive myenteric region. To test if these undifferentiated NCCs of Shh-treated explants could migrate in response to GDNF, we pretreated explants with or without Shh for 2 d, then replaced the medium with GDNF containing medium, and cultured for another 2 d. Shh pretreatment increased the number of migratory cells by twofold ( $P = 0.035$ ,  $t$  test) as compared with those pretreated with the control medium (Fig. 7 I). Distributions of Shh in Shh-treated

and control explants of *b3-IIIa-LacZ* mice were analyzed by immunofluorescence. The Shh immunofluorescence signal in control explant (Fig. 7 K) was strong at the mucosa, whereas the signal at the mesenchyme declined the further it was away from the mucosa, which was similar to that of mouse embryonic gut (compare Fig. 7 K with Fig. 1 L). The fluorescence signal at the gut mesenchyme (Fig. 7, asterisk) of the control explant was the auto-fluorescence of  $\beta$ -galactosidase-expressing NCCs (Fig. 7, blue) at the presumptive myenteric region (compare Fig. 7 K and Fig. 7 L). A strong Shh immunofluorescence signal was detected across the gut radius at the mucosa, mesenchyme, and serosa in Shh-treated explants (Fig. 7 J, arrowheads). This finding indicated that Shh treatment disrupted the endogenous Shh gradient across the gut radius in which Shh concentration declined from the mucosa toward the serosa. Staining without 5E1 or with mouse IgG isotype control antibody gave no signal (Fig. 7, M and N).

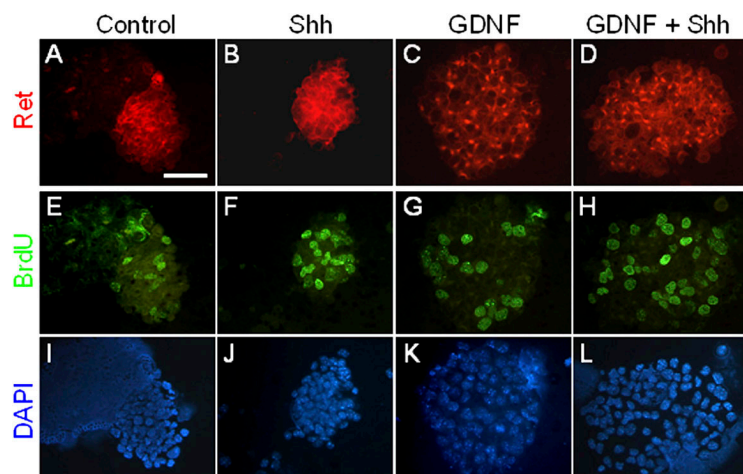
Immunofluorescence was performed on cytospin preparations of mildly dissociated explants that were cultured in the presence of BrdU. Cell clumps were identified as clusters of Ret+ cells (Fig. 8, A–D), and proliferating cells were localized with anti-BrdU antibody (Fig. 8, E–H). Culturing explants for 3 d in different treatments did not affect the percentage of Ret+ cells in cell clumps (Table IV). However, treatments with Shh or Shh plus GDNF increased the percentage of Ret+/BrdU+ cells in cell clumps by nearly twice as much as those in control explants and explants treated with GDNF alone (Table IV).

## Discussion

We have shown that Shh promotes proliferation but inhibits neuronal differentiation of enteric NCCs and restricts GDNF-induced NCC migration. Shh may regulate the development of neural plexuses by modulation of the responsiveness of NCCs toward GDNF inductions and/or regulation of the proliferation and differentiation of NCCs in gut.

### Enteric NCCs in neurospheres remain multipotent

Multipotent enteric NCCs migrate from foregut to hindgut between E9.5 and E13.5 in mouse (Kapur et al., 1992; Dur-



**Figure 8. Shh promotes the proliferation of NCCs in gut explants.** Explants cultured in different treatments with BrdU for 3 d were mildly triturated and cytospun onto microscope glasses for staining with anti-Ret (A–D, red) and anti-BrdU (E–H, green) antibodies. (I–L) DAPI-stained nuclei of cell clumps in various treatments are shown. All photos were taken at the same magnification. Bar, 25  $\mu$ m.

bec et al., 1996; Young et al., 1998; Natarajan et al., 2002). During migration in gut mesenchyme, NCCs proliferate and differentiate into neurons and glia. We reasoned that E11.5 mouse gut contained proliferative and undifferentiated NCCs. Our data indicated that neurospheres prepared from E11.5 mouse guts contained proliferating NCCs. Neurospheres could be replated at clonal density. Furthermore, neurospheres contained neurons, glia, and myofibroblasts. These findings indicate that NCCs of neurospheres display self-renewal properties and have the potentials for differentiation into progenitors of multi-lineages, which are characteristic features of gut neural crest stem cells (Morrison et al., 1999, 2000; Bixby et al., 2002; Barlow et al., 2003; Bondurand et al., 2003; Kruger et al., 2003).

### Shh and GDNF display opposite functions in the regulation of proliferation and differentiation of enteric NCCs

In the presence of Shh, neuronal differentiation of neurospheres was minimal and neurospheres contained abundant proliferating NCCs. NCC clusters were bigger with more proliferating NCCs in the presence of Shh than those in its absence and in GDNF culture. GDNF-treated clusters contained prominent neuronal processes, indicating that GDNF promotes NCC differentiation. Apoptotic cells were rarely seen in cultures with or without Shh. Together, these findings indicate that Shh promotes the proliferation and inhibits the differentiation of NCCs. NCC clumps in the explants treated with Shh, and in those treated with Shh plus GDNF, contained twice as many Ret<sup>+</sup>/BrdU<sup>+</sup> cells as those in plain medium and GDNF-treated explants. These findings indicate that Shh represses the neuronal induction of GDNF on NCCs. GDNF displays different effects on enteric NCCs, where it promotes the proliferation of neuron and glia progenitors at low concentration but promotes the glial differentiation at high concentrations (Heuckeroth et al., 1998; Ganino et al., 2003). The lack of a proliferation promotion of GDNF in our work could be attributed to differences in culture conditions and methods of preparation of NCCs between our work and others. However, GDNF could have dual functions in our explant where it promotes NCC proliferation in the early phase of culture but promotes neuronal

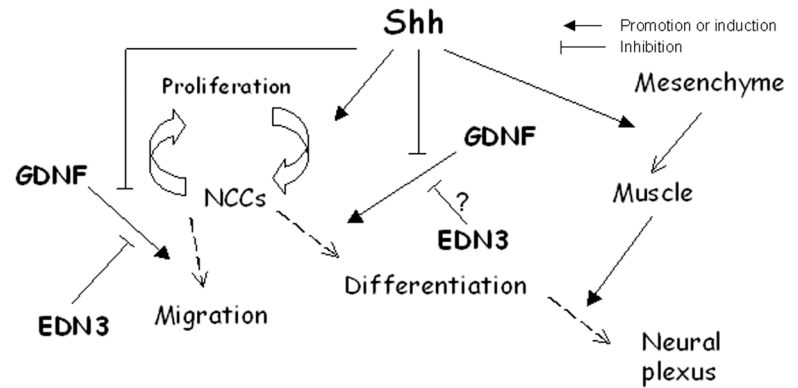
differentiation at the later culture period. The high number of Ret<sup>+</sup> cells with relatively low BrdU incorporation in cell clumps of GDNF-cultured explant is in line with such dual effects of GDNF in explant. The high number of Ret<sup>+</sup> cells is probably originated from NCC proliferation in the early phase of GDNF culture, and NCCs underwent neuronal differentiation in the later culture period.

### Regulatory roles of Shh in the development of neural plexuses in gut

Consistent with previous data (Young et al., 2001), we also showed that the NCC migration from explant is GDNF dependent. GDNF-induced NCC migration from explant was inhibited by Shh. Inhibition of NCC migration along the hindgut by Shh was also observed if the whole gut was cocultured with GDNF and Shh; and cell clumps were frequently observed on explant (Fig. S1, A–H, available at <http://www.jcb.org/cgi/content/full/jcb.200401077/DC1>). GDNF binds to the receptor complex of Ret and Gfra-1 on NCCs, and Ret expression by NCCs is essential for GDNF-induced migration (Taraviras and Pachnis, 1999; Taraviras et al., 1999). Ret expression of NCCs in gut explants and of those migrated onto the filter appeared unaffected by Shh. Apparently the inhibition of NCC migration by Shh is not mediated by abolishing the expression of GDNF receptor complex on NCCs. GDNF can bind directly to neural cell adhesion molecule (NCAM) stimulating Schwann cell migration and axonal growth of neurons (Paratcha et al., 2003). Shh cultured neurospheres did not express NCAM, but neurospheres cultured with GDNF expressed a high level of NCAM. Furthermore, differentiated NCCs expressed Ret and NCAM, whereas undifferentiated NCCs expressed Ret only in Shh-treated explants with or without GDNF (Fig. S1, I–L). Lack of NCAM on NCCs in Shh GDNF cotreated explant could explain at least in part the lack of migration and differentiation of NCCs in response to GDNF. Shh may directly or indirectly regulate NCAM expression of NCCs and modulates the response of NCCs to GDNF.

Neural plexus failed to form properly in the gut at either too high (in Shh-treated explants) or too low Shh concentrations (in *Shh*<sup>-/-</sup> mice; Ramalho-Santos et al., 2000),

**Figure 9. Shh regulates multiple aspects of growth of NCCs in the gut.** Shh, GDNF, and EDN3 act together to orchestrate the development and the concentric organization of NCCs and musculature of the developing gut. Shh promotes the proliferation and inhibits the differentiation of NCCs. Shh suppresses the pro-neurogenic effects of GDNF on NCCs. Shh and EDN3 restricts the GDNF-induced NCC migration. Shh induces muscle differentiation of mesenchyme, which in turn affects the neural plexus formation.



indicating that an optimal Shh level is critical for plexus formation. Shh is synthesized in the gut endoderm and secreted into the gut mesenchyme forming a Shh gradient across the gut radius. Ptc1 was localized throughout the E11.5 and E13.5 gut mesenchyme. By binding to Ptc1 on mesenchyme cells, Shh regulates the differentiation of smooth muscle layers (Roberts et al., 1995; Sukegawa et al., 2000; Gustafsson et al., 2002). The muscle layers were not developed properly, and the myenteric region was not demarcated in Shh-treated explants. Development of the myenteric plexus correlated with the differentiation of smooth muscle layers and the spatio-temporal distributions of ECM molecules and cell adhesion molecules (Newgreen and Hartley, 1995). Thus, abnormal plexus formation in Shh-treated explants could be the consequence of aberrant mesenchyme differentiation. It has been shown that Shh arrests the migration of sensory precursors regulating the position of trigeminal ganglia in cranial mesenchyme by the induction of the expression of proteoglycan versican in the mesoderm (Fedtsova et al., 2003). Expression study of ECM molecules in Shh-treated explants will shed light on the functions of Shh on mesenchyme differentiation and neural plexus formation.

In the neurosphere, Ptc1 immunoreactivity overlapped with that of Ret, and cells showing intense Ptc1 immunoreactivity were negative for SMA. We reasoned that Ret<sup>+</sup> cells that coexpressed Ptc1 in neurosphere were NCCs and neuron progenitors. Furthermore, NCCs of mouse embryonic gut expressed Ptc1. These findings raise the possibility that Shh could bind to Ptc1 on NCCs, regulating their growth. Binding of Shh to Ptc1 restricts the movement of Shh and sculpts the Shh gradient in the neural tube, suggesting a direct long-range morphogen activity of Shh (Briscoe et al., 2001). The expression of Ptc1 in the mesenchyme could pattern the Shh concentration across the gut radius, which in turn could regulate the neural plexus formation by controlling the proliferation, differentiation, and coalescence of NCCs. However, the regulation of Shh on plexus formation by indirect mechanism (i.e., Shh regulates the mesenchyme, which in turn modulates the growth of NCCs) and direct mechanism (i.e., Shh binds to Ptc1 on NCCs, regulating their growth and/or responsiveness toward mesenchyme-derived factors) are not necessarily mutually exclusive. Conditional knockout of *Ptc1* in enteric NCCs in mice would clarify the functional significance of direct Shh regulation on NCCs.

Besides GDNF, mesenchyme-derived factor EDN3 also plays an important role in controlling the growth of enteric NCCs (for review see Chakravarti and Lyonnet, 2001). GDNF promotes the proliferation, differentiation, and migration of enteric NCCs, and EDN3 inhibits their differentiation (Hearn et al., 1998; Heuckeroth et al., 1998; Taraviras et al., 1999; Wu et al., 1999; Worley et al., 2000; Young et al., 2001; Focke et al., 2003; Gianino et al., 2003). In contrast to Shh's proliferative effect on NCCs, EDN3 alone has no obvious proliferative effect on NCCs (Barlow et al., 2003). Interestingly, EDN3 strongly enhances the proliferation of NCCs in the presence of GDNF. However, EDN3 displays similar effects as Shh in the inhibition of GDNF-induced NCC migration (Barlow et al., 2003; Kruger et al., 2003). Temporal requirement of EDN3/EDNRB signaling in the development of NCCs in gut has also been demonstrated (Shin et al., 1999; Kruger et al., 2003), suggesting that the responsiveness of NCCs toward inductions of mesenchyme-derived factors is developmentally regulated. Investigation of NCC development in mice with compound deficiencies of Shh signaling, EDN3/EDNRB signaling, and/or GDNF/Ret signaling will be important in understanding the combined effects of these factors in ENS development.

In conclusion, gut endoderm-derived Shh and mesenchyme-derived factors act together to orchestrate the development and the concentric organization of NCCs and musculature of developing gut (Fig. 9). Furthermore, our data show that NCCs can be maintained in culture without losing stem cell properties in the presence of Shh. The practicality of using Shh in the isolation of NCCs for cell replacement therapy for the treatment of NCC-associated anomalies deserves further investigation.

## Materials and methods

### Neurosphere culture

E11.5 ICR mouse guts (from stomach to hindgut) were dissected in L15 medium. Guts were washed with Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free PBS digested with collagenase and dispase (0.2 mg/ml each; 37°C for 10 min). Digested guts were triturated into single cells and filtered through cell strainers (80  $\mu$ m and then 40  $\mu$ m), washed with L15, resuspended in NCC medium (Natarajan et al., 2002), and plated onto poly-D-lysine- and fibronectin (20  $\mu$ g/ml each)-coated wells. Mesenchyme cells grew as a monolayer, and NCCs formed clusters. Cultures were replated ( $8 \times 10^4$  cells per each 35-mm well) once every 40–50 h, and very few mesenchyme cells remained in culture after five passages. NCC clusters increased in size, formed domes, and developed into neurospheres. Neurospheres could be maintained in culture by passage once every 4–5 d. 2  $\mu$ g/ml Shh (recombinant mouse



Shh-N; R&D Systems) and 50 ng/ml GDNF (recombinant human GDNF; PeproTech) were added either alone or in combination after passage. Neurospheres could also be replated at low and clonal density (500 cells per each 35-mm dish), and neurospheres were reformed after 5–7 d.

### Dissociated gut culture

Single cell suspensions equivalent of 10 E11.5 ICR mouse guts were seeded onto each 35-mm well and cultured overnight in NCC medium. 2  $\mu$ g/ml Shh and 50 ng/ml GDNF were added either alone or in combination on the first day of culture. Culture medium was changed once every 2 d.

### Gut explant culture

E11.5 guts of *b3-IIIa-LacZ* mice or ICR mice were dissected. A thin layer of 2.5% (wt/vol) agarose gel in PBS with penicillin and streptomycin (100 U/ml) was laid onto the well. Cutting end at the cecum was placed onto the filter membrane (0.45  $\mu$ m; Black Gridded HABG; Millipore), and the proximal end was secured to the agarose bed with a 4–0 steel line (ETHICON). Explant was cultured in DME supplemented with 10% FCS and antibiotics. 100 ng/ml GDNF and 2  $\mu$ g/ml Shh were added either alone or in combination. NCCs migrated onto the filter after culturing, and the number of migratory cells was counted by flow cytometry. For counting, explants with the filter were transferred to a new dish; explants were carefully removed from the filter without dislodging the cells from the filter. The cells were released from the filter by incubation with 300  $\mu$ l of Trypsin (37°C for 20 min). Cells were fixed by the addition of 300  $\mu$ l of 4% PFA/PBS. Cells were counted on a flow cytometer (FACS Calibur; Becton Dickinson) or were cytospun onto TESPA (3-aminopropyl-triethoxy-silane; Sigma-Aldrich)-coated microscope glass for immunohistochemistry.

### Immunofluorescence

Immunofluorescence analyses were performed to localize NCCs and their derivatives using antibodies that recognized antigens of NCCs, neurons, glia, myofibroblasts, proliferative cells, and cells expressing Ptc1 (Table S1, available at <http://www.jcb.org/cgi/content/full/jcb.200401077/DC1>). Processing of cultures/cells/sections and immunostaining were detailed as described in the online supplemental materials. To ascertain the specific staining of antibody, cultures/cells/sections were incubated with blocking solution without primary antibody or with isotype control antibody. Weak nonspecific signal was detected if primary antibody was omitted or isotype control antibody was used.

### $\beta$ -galactosidase activity staining

Embryos or explants with filters were fixed in PFA/PBS (1 h for embryo; 20 min for explants) at RT. X-gal/IPTG staining was performed as describe previously (Sanes et al., 1986). Stained embryos or explants were fixed and processed for paraffin embedding. 6- $\mu$ m-thick sections were prepared and mounted onto TESPA-coated microscope glasses. Antigen retrieval and immunohistochemistry were performed as described previously (Fu et al., 2003). Signal was developed using EnVision System, HRP (Dako). Sections were mounted in aqueous mounting medium (Dako).

### BrdU incorporation and immunohistochemistry

BrdU incorporation was performed using in situ Cell Proliferation kit, Fluos (Roche). BrdU was added 4 h before the termination of culture. Culture was fixed, blocked, and incubated with anti-Ret antibody. Genomic DNA was denatured in 4 N HCl (40 min at RT). After PBS washing, the cultures were incubated with fluorescein-conjugated anti-BrdU antibody (Roche) and a TRITC-conjugated rabbit anti-goat antibody (37°C for 1 h). Slides were washed in PBS, dehydrated, dried, and mounted with anti-fade mountant with DAPI.

### TUNEL

Apoptotic cells in neurosphere were analyzed on cytospin preparation of neurospheres using in situ Cell Death Detection kit, Fluorescein (Roche), following the manufacturer's protocol.

### Microscopic analysis and image capture

Cultures/cells/sections were examined with a confocal microscope (BioRad Laboratories), an epifluorescence microscope (Nikon), or a dissection microscope (model MZFLIII; Leica). Images were captured with digital camera (Nikon) and photos were compiled using Adobe Photoshop 7.

### In situ hybridization

Section in situ hybridization using DIG-labeled riboprobes were performed as described previously (Schaeren-Wiemers and Gerfin-Moser,

1993; Wilkinson and Nieto, 1993). Riboprobes were synthesized using DIG RNA labeling kit (Roche). Hybridization signal was amplified with Tyramide Signal Amplification system and Streptavidin-HRP conjugate following the manufacturer's protocol (TSA Biotin System; PerkinElmer). Sections were stained in hematoxylin, dehydrated, cleared in xylene, and mounted in DPX mountant (BDH). The probes used were *Shh* (Echelard et al., 1993), *Ptc1* (Goodrich et al., 1996), *Gli1* (Hui et al., 1994), and *Ret* (Pachnis et al., 1993).

### Statistical analysis

*t* test was performed for paired samples, and ANOVA with Tukey post-hoc corrections was performed for multiple comparisons. The significant level was set at 5%.

### Ptc1 antibody

The anti-Ptc1 antibody was provided by B. Wainwright (Institute for Molecular Bioscience, The University of Queensland, St. Lucia, Australia).

### Online supplemental material

Protocols of the processing of cultures/cells/sections and immunostaining and a list of specific primary antibodies that were used in this work are available at <http://www.jcb.org/cgi/content/full/jcb.200401077/DC1>.

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