MATERIAL AND METHODS

Isolation and identification of pNCSCs

Neural tubes were isolated from the trunk level using mouse embryos (9 day post coitus, dpc), and the pNCSCs were isolated from the NT following the established procedure, with some modifications \(^1\). Briefly, trunk sections of NT were treated with 1mg/mL collagenase IV (172 U/mg, Gibco, Grand Island, NY, USA) and 500 ug/ml Dispase II (0.77 U/mL, Gibco) in DMEM (Gibco) for 4-6 min at room temperature. Thereafter, the tissues surrounding the NT were gently cut off with needles and washed twice with DMEM. The NT explants were plated into 6-well Corning plates (Corning, NY, USA) coated with Poly-L-ornithine (Sigma, St. Louis, MO, USA), and cultured in pNCSCs Proliferation Medium at 37\(^\circ\)C, in 5% CO2. The proliferation medium was made in 500 mL batches containing 432-mL DMEM:F12, 50-mL bovine serum albumin (20% (vol/vol) stock solution, Millipore, Bedford, MA, USA, 82-100-5), 5 mL of penicillin/streptomycin (Gibco), 5 mL of l-alanyl-l-glutamine (Cellgro, Herndon, VA, United States), 5 mL of MEM non-essential amino acids (Cellgro), 0.5 mL of trace elements A (Cellgro), 0.5 mL of trace elements B (Cellgro), 0.5 mL of trace elements C (Cellgro), 0.5 mL of 2-mercaptoethanol (Invitrogen), transferrin (10 mg/mL, Invitrogen), (+) sodium l-ascorbate (50 mg/mL, Sigma), Heregulin B-1 (10 ng/mL, Peprotech, Rocky Hill, NJ, USA), LONGR3 (200 ng/mL, Sigma), bFGF (8 ng/mL, Peprotech), and 10 mL of fetal bovine serum (Gibco). In order to identify the pNCSCs migrating from the NT, the NT isolated from the EGFP\(^+\) mice were cultured on glass slides, and the pNCSCs were initially identified and selected following the expression of P75NTR (hereafter referred to just as P75) by immunostaining after 48 h.
Electrophysiology

For in vitro electrophysiology recordings, the pNCSCs were plated onto small round glass coverslips at 37°C, 5% CO2. The cells were visualized using an Olympus Optical Microscope (BX51WI, Tokyo, Japan) with differential interference contrast optics at 40× powers. Whole-cell patch recording was performed to examine the electrophysiology properties of pNCSC-derived neurons. The EGFP+ cells derived from the populated colon at 3-5 wk after transplantation were analyzed for electrophysiology. For the electrophysiology recording of EGFP+ cells in the recipient's colon, the muscle strips containing EGFP+ cells were isolated from the recipient's colon and treated with collagenase IV. Subsequently, the derived EGFP+ cells were attached to coverslips for further electrophysiology tests. The coverslips were transferred to a recording dish that was continuously perfused with artificial cerebrospinal fluid (ACSF) saturated with 95% O2/5% CO2. The composition of ACSF was (in mM) 124 NaCl, 3.5 KCl, 1.5 CaCl2, 1.3 MgSO4, 1.24 KH2PO4, 18 NaHCO3, and a lot of glucose, at pH 7.4. Electrodes were pulled from glass capillaries using a Sutter instrument puller (model P-97). The electrodes were filled with a solution consisting of (in mM) 140 K-gluconate, 0.1 CaCl2, 2 MgCl2, 1 EGTA, 2 ATP K2, 0.1 GTP Na3, and 10 HEPES, with a pH of 7.25 (290 mOsm) and having a resistance of 4-6 MΩ. Whole-cell voltage-clamp or current-clamp recordings were performed at 30°C.

Voltage and current-clamp recordings were obtained using a MultiClamp 700B amplifier (Axon Instruments, San Jose, CA, USA). The signals were filtered at 4 kHz using a Digidata 1322A analog-to-digital converter (Axon instruments). Access resistance was monitored before
and after the recordings and cells with resistances of > 25 MΩ at either point was discarded from the analyses. All the data were obtained from independent coverslips. Offline data analysis was performed using Clampfit 9.0 (Axon) and Origin (Origin).

**Transplantation into the adult mouse colon**

The surgical procedure for transplantation followed that reported previously. Premigratory NCSCs at P3 or P4 were transplanted into the distal colon of 6-8-week-old C57BL/6J wild type or NOD/SCID mice. At 1-18 wk following surgery, the mice were sacrificed, and the tissue was collected for histological analysis.

**Generation of the human tissue-engineered intestine with pNCSCs**

A Human ES cell line H1 was maintained on a Matrigel in mTeSR (STEMCELL Technologies, Shanghai, China, 05850) medium and routinely passaged with collagenase IV (Gibco). HIOs were generated as described previously. Briefly, hESCs line H1 was first induced into the definitive endoderm (DE) by treatment with activin A (Novoprotein, Shanghai, China) for 3 d. DE was further induced into three-dimensional hindgut spheroids with Chir99021 treatment (3 µM, Boston, MA, USA) and FGF4 (500 ng/mL, Novoprotein) 4 d. Spheroids were cultured in EGF (500 ng/mL) in three-dimensional conditions to form human intestinal organoids (HIOs). We mixed one HIO (at 14-20 d of differentiation) with 60,000–100,000 pNCSCs in Matrigel that was then cultured in a Basic Gut Medium (Advanced DMEM/F12, 1× B27 supplement, 1× N2 supplement, 10 µM HEPES (Invitrogen), 2 mM l-glutamine, 1 × Pen-Strep, and 100 ng/mL EGF). Growth was maintained in vitro for 7-10 d. For TEI maturation, HIOs
and HIOs + pNCSCs were transplanted into the kidney capsule of BALB/c nude mice following the previous protocol \(^4,5\), and allowed to grow for 6-10 wk to \textit{in vivo} maturity.

**Immunoochemistry**

Immunostaining of 10-12 µm thick frozen cryosections was performed using the intestines of each mouse or HIO. The distal colon was opened along the mesenteric border, pinned, stretched on Sylgard coated dishes, fixed in 4% paraformaldehyde, and dehydrated in 30% sucrose with phosphate buffer saline (PBS). Thereafter, it was transferred to an O.C.T. cryomold (Tissue-Tek). For the mouse colon, frozen sections were cut transversely or longitudinally on a cryostat. Immunofluorescence staining was performed on cultured cells, spheres, and cryosections of intestinal samples. Cultured cells or tissue sections were fixed with 4% PFA for 15 min at room temperature (RT) and rinsed using PBS and exposed to blocking solution (PBS containing 5% animal serum and 0.2% Triton X-100) for 30 min at RT followed by incubation with the primary and secondary antibodies.

Whole-mount immunostaining was performed on the natural and TEI intestinal segments following excision and removal of the mucosa via sharp dissection. Tissues were fixed in 4% paraformaldehyde for 24-48 h at 4°C. After fixation, the tissues were washed for 24 h in PBS at 4°C. The tissues were blocked for 1 h (PBS containing 0.3% Triton X-100, 5% donkey serum) and then incubated in primary antibody (diluted in PBS containing 0.3% Triton X-100, 5% donkey serum) for 36 h at 4°C or 3.5 h at room temperature (about 25°C), and immunoreactivity was detected using secondary antibodies (1:500 in PBS 1 h at room temperature). Before mounting, the tissues were thoroughly washed with PBS for 2 h at room temperature or 12 h at
4°C. The primary and secondary antibody information is listed in Table 1 and Table 2, respectively. The tissues were examined using a Nikon A1 confocal microscope (Nikon, Japan).

Immunostaining were performed for each marker at least three times.

**Table1, primary antibody list**

| Antigen | Supplier | Cat. NO | Host | Dilution | RRID   |
|---------|----------|---------|------|----------|--------|
| P75NTR  | Abcam    | ab8875  | Rabbit | 1:200 | AB_306828 |
| Nestin  | Millipore | MAB353  | Mouse  | 1:200  | AB_94911  |
| AP2     | Santa Cruz | sc-12726 | Mouse  | 1:50   | AB_667767  |
| SOX10   | Abcam    | ab155279 | Rabbit | 1:250  | AB_2650603 |
| Ki67    | Abcam    | ab15580  | Rabbit | 1:500  | AB_443209  |
| TuJ1    | Covance  | mrb-435p | Rabbit | 1:200  | AB_663339  |
| TuJ1    | Promega  | G712A   | Mouse  | 1:500  | AB_430874  |
| PGP9.5  | Abcam    | ab8189  | Mouse  | 1:200  | AB_306343  |
| NF200   | Sigma    | N4142   | Rabbit | 1:200  | AB_477272  |
| GFAP    | Abcam    | ab7260  | Rabbit | 1:200  | AB_305808  |
| S100β   | Abcam    | ab52642 | Rabbit | 1:200  | AB_882426  |
| nNOS    | Abcam    | ab76067 | Rabbit | 1:500  | AB_2152469 |
| VIP     | Santa Cruz | sc-20727 | Rabbit | 1:20   | AB_2304501 |
| ChAT    | Millipore | AB144p  | Goat   | 1:50   | AB_2079751 |
| ChAT    | Proteintech | 20747-1-AP | Rabbit | 1:100  | AB_10898169 |
| Antibody     | Supplier  | Cat.No | Dilution | RRID       |
|--------------|-----------|--------|----------|------------|
| HuC/D        | Abcam     | ab184267 | Rabbit   | 1:500      | AB_2864321 |
| Synapsin-1   | CST       | 5297   | Rabbit   | 1:100      | AB_2616578 |
| SMA          | Abcam     | ab5694  | Rabbit   | 1:1500     | AB_2223021 |
| C-KIT        | Abcam     | ab32363 | Rabbit   | 1:100      | AB_731513  |
| CDX2         | Abcam     | ab76541 | Rabbit   | 1:500      | AB_1523334 |
| CDH1         | R&D       | AF648   | Goat     | 1:20       | AB_355504  |
| FoxA2        | Abcam     | ab108422 | Rabbit   | 1:300      | AB_11157157 |
| Sox17        | R&D       | AF1924  | Rabbit   | 1:20       | AB_355060  |

Table 2, secondary antibody list

| Secondary antibody                                                                 | Cat.No | Dilution | supplier                        | RRID       |
|-----------------------------------------------------------------------------------|--------|----------|---------------------------------|------------|
| Donkey anti-Mouse IgG (H+L), Alexa Fluor 488                                      | A-21202 | 1:500    | Thermo Fisher Scientific        | AB_141607  |
| Donkey anti-Rabbit IgG (H+L), Alexa Fluor 488                                     | A-21206 | 1:500    | Thermo Fisher Scientific        | AB_2535792 |
| Donkey anti-Rabbit IgG (H+L), Alexa Fluor 594                                     | A-21207 | 1:500    | Thermo Fisher Scientific        | AB_141637  |
| Donkey anti-Mouse IgG (H+L), Alexa Fluor 594                                      | A-21203 | 1:500    | Thermo Fisher Scientific        | AB_141633  |
| Donkey anti-Goat IgG (H+L), Alexa Fluor 594                                       | A-32758 | 1:500    | Thermo Fisher Scientific        | AB_2762828 |
| Donkey anti-Rabbit IgG (H+L), Alexa Fluor 647                                     | A-31573 | 1:500    | Thermo Fisher Scientific        | AB_2536183 |

Supplemental Figures
Supplemental Figure 1. Premigratory NCSCs generate neural and mesenchymal lineage when cultured in Differentiation Medium I for 10-14 d. Terminal differentiation of EGFP$^+$ cells into neurons and glia cells (A) are recognized by antibodies of the neuronal marker TuJ1 (a neuronal marker) and GFAP (an enteric glial and astrocyte marker). Terminal differentiation into smooth muscle cells is identified via the immunostaining of smooth muscle actin$^+$ (SMA, a smooth muscle marker) (B). Nuclei are counter-stained with DAPI. Scale bars, 100 μm.

Supplemental Figure 2. The Ki67 positive (67.7% ± 8.7%) immunostained cells at P3 in the culture are shown. Scale bars, 100 μm.
Supplemental Figure 3. Most cells maintain the expression of NSCs marker Nestin (A, 94.33% ± 3.37%) and NCSCs markers P75 (B, 93.27% ± 2.58%), SOX10 (C, 92.50 ± 2.95%) and AP2 (D, 85.3 ± 2.10%) at P4, about 20 d after proliferation in the proliferation medium. Scale bars, 100 μm.
Supplemental Figure 4. Premigratory NCSCs-derived cells are mainly distributed in the muscle layer and preliminarily differentiate into the neuron and glia-like cells 3 weeks following transplantation. (A-D) The co-localization of EGFP+ fluorescence with TuJ1 staining in the three-dimensional picture indicate that most grafted cells survived and differentiated into TuJ1 positive cells. The EGFP+ cells produce a dispersed distribution, and connect with each other in the mouse colon (A and B). (C) The immunostaining of the transverse section displays the distribution of exogenous pNCSCs-derived neurons. EGFP+ cells are observed in the mouse colon wall, mainly localized between the LM and CM layers. A few EGFP+ fibers extended to
the mucosa and submucosa layers. (D) Part of EGFP$^+$ cells are stained as S100 β-positive cells.

Sections of mouse colon are counterstained in blue with DAPI to identify the cell nuclei. Scale bars, (A), 200 µm, (B-D) 100 µm.

**Supplemental Figure 5.** (A) Immunostaining of endoderm markers, including FOXA2 and SOX17, showing the induction of endoderm from hESCs. (B) The immunostaining result of epithelium marker CDH1 and CDX2, showing the differentiation of epithelium during the stage of hindgut induction from hESCs.

**Supplemental Figure 6.** The negative immunostaining of TuJ1 and S100β is showed
individually in the left image of (A) and (B) which displays the cross-section of myenteric and submucosal layers in Ctrl-TEI samples without pNCSCs. The immunostaining of neuronal marker NF-L and glia cell marker S100β is displayed in the left image of (C) and (D) which shows the section of pNCSCs-TEI. In the right, it shows the merged image with DAPI in (A-D). Scale bars, (A-B) 100 µm, (C-D) 50 µm.

**Supplemental video 1.** Control TEI demonstrates spontaneous phasic contraction and relaxation.

**Supplemental video 2.** TTX treatment shows no obvious change compared with the spontaneous phasic peristalsis in Ctrl-TEI.

**Supplemental video 3.** Treatment with methylene blue abolishes the peristalsis in Ctrl-TEI.

**Supplemental video 4.** Premigratory NCSCs-TEI demonstrates spontaneous phasic contraction and relaxation.

**Supplemental video 5.** Following the treatment with methylene blue, pNCSCs-TEI shows a reduction in contractility instead of the complete blocking.

**Supplemental video 6.** Treatment of pNCSCs-TEI with Tetrodotoxin evidently inhibits the peristalsis during the inspection period.

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