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°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% O_2/5% CO_2. The composition of ACSF was (in mM) 124 NaCl, 3.5 KCl, 1.5 $CaCl_2$, 1.3 $MgSO_4$, 1.24 KH_2PO_4, 18 $NaHCO_3$, 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 $CaCl_2$, 2 $MgCl_2$, 1 EGTA, 2 ATP K_2, 0.1 GTP Na_3, 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 [2]. 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 [3, 4]. 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 *in vivo* maturity.

Immunochemistry

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

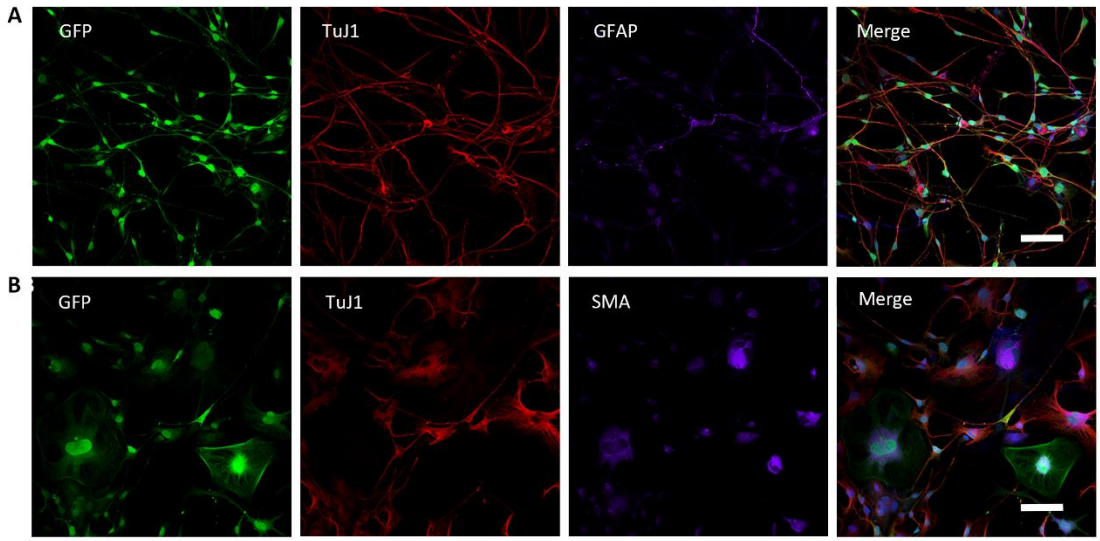
Antigen	Supplier	Cat. NO	Host	Dilution	RRID
P75NTR	Abcam	ab8875	Rabbit	1:200	AB_306828
Nestin	Millipore	MAB353	Mouse	1:200	AB_94911
AP2	Santa Cruz	sc-12726	Mouse	1:50	AB_667767
SOX10	Abcam	ab155279	Rabbit	1:250	AB_2650603
Ki67	Abcam	ab15580	Rabbit	1:500	AB_443209
TuJ1	Covance	mrb-435p	Rabbit	1:200	AB_663339
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

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

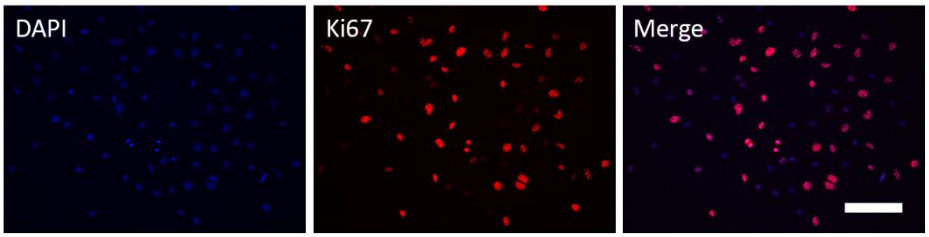
Table 2, secondary antibody list

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

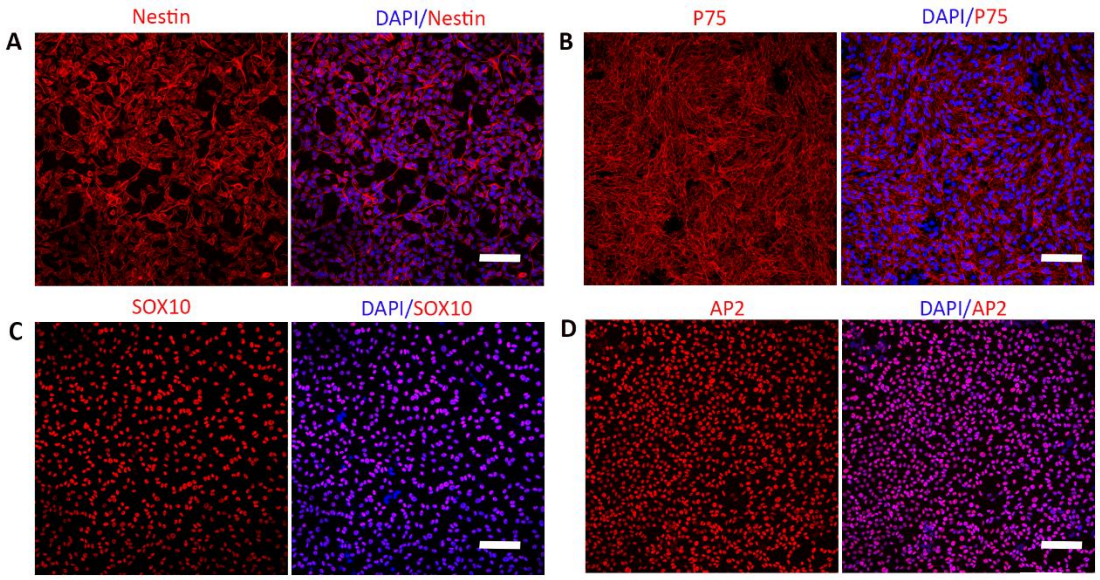
Supplemental Figures



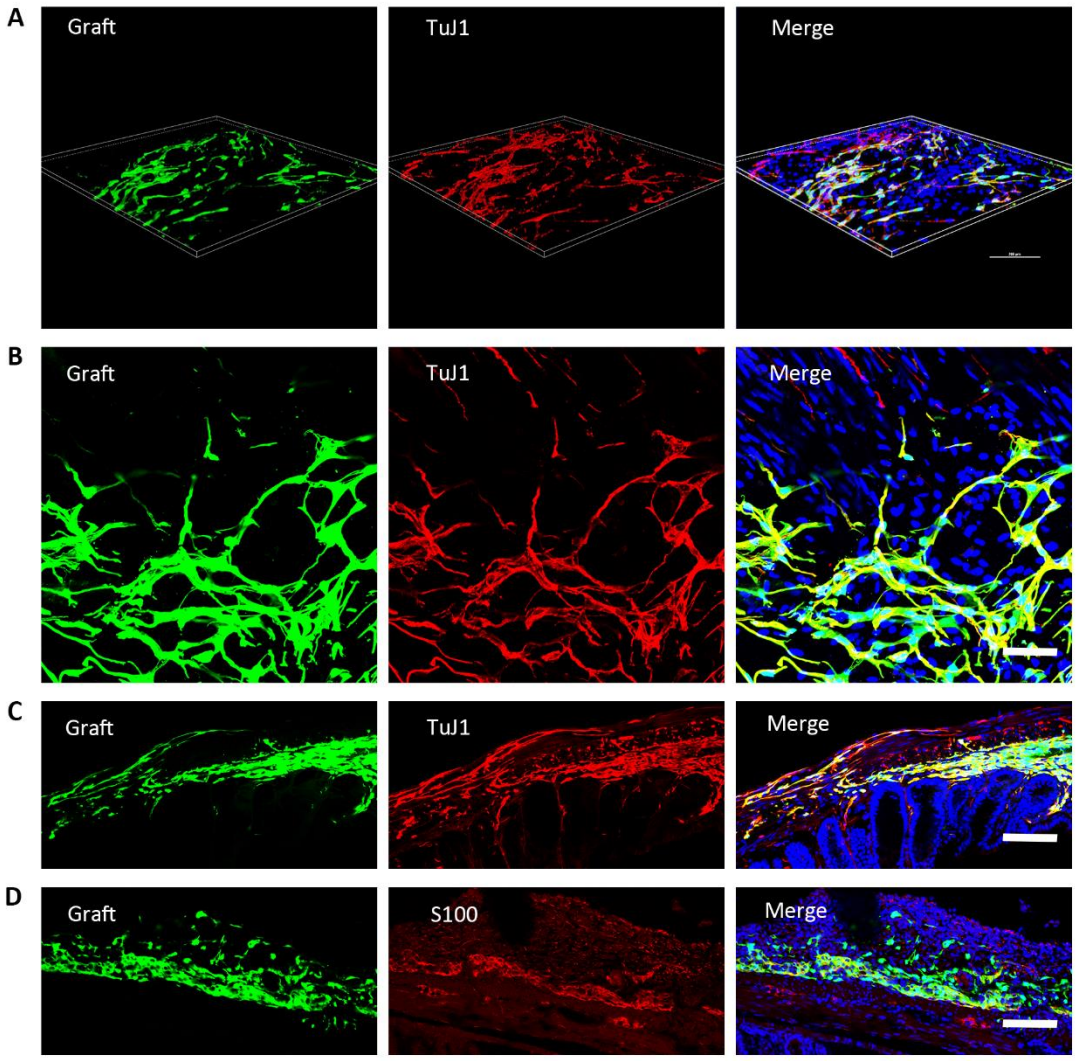
Supplemental Figure 1. Premigratory NCSCs generate neural and mesenchymal lineage when cultured in Differentiation Medium I for 10-14 d. Terminal differentiation of $EGFP^+$ cells into neurons and glia cells (A) are recognized by antibodies of the neuronal marker TuJ1 (a neuronal marker) and GFAP (an 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.

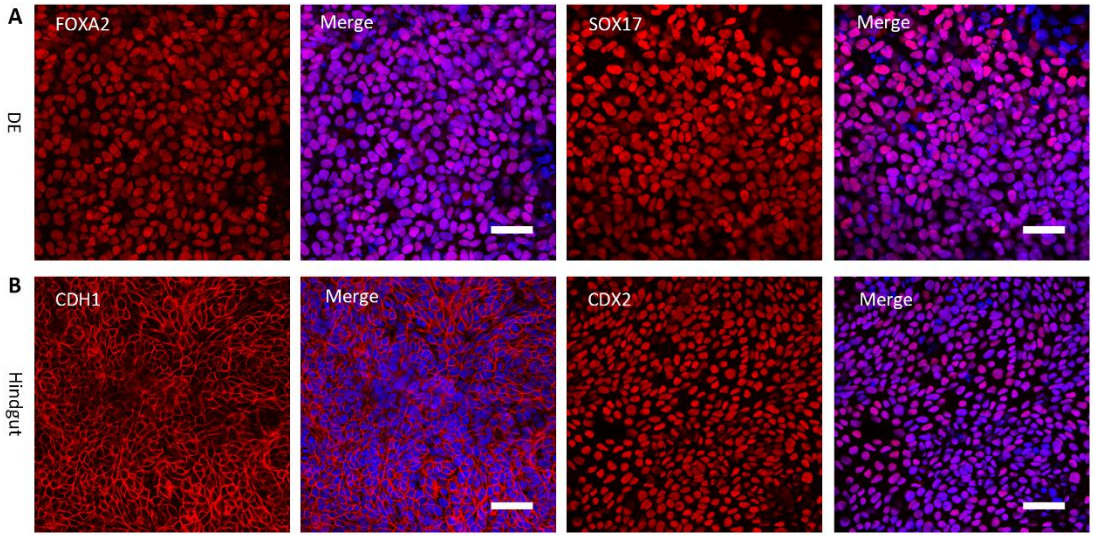


Supplemental Figure 3. Most cells maintain the expression of NSCs marker Nestin (A, 94.33% ± 3.37%) and NCSCs markers P75 (B, 93.27% ± 2.58%), SOX10 (C, 92.50 ± 2.95%) and AP2 (D, 85.3 ± 2.10%) at P4, about 20 d after proliferation in the proliferation medium. Scale bars, 100 μm.

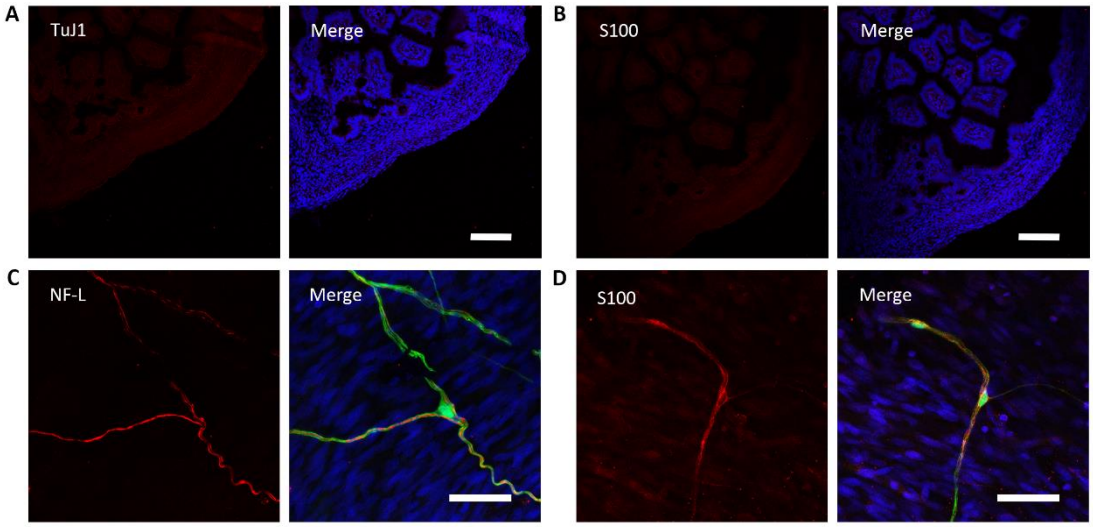


Supplemental Figure 4. Premigratory NCSCs-derived cells are mainly distributed in the muscle layer and preliminarily differentiate into the neuron and glia-like cells 3 weeks following transplantation. (A-D) The co-localization of $EGFP^+$ fluorescence with TuJ1 staining in the three-dimensional picture 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 ß-positive cells. Sections of mouse colon are counterstained in blue with DAPI to identify the cell nuclei. Scale bars, (A), 200µm, (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 S100ß 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.

RERERENCE

1 Etchevers H. Primary culture of chick, mouse or human neural crest cells. *Nature protocols* 2011; **6**:1568-1577.

2 Hotta R, Stamp LA, Foong JP *et al.* Transplanted progenitors generate functional enteric neurons in

the postnatal colon. *The Journal of clinical investigation* 2013; **123**:1182-1191.

3 Spence JR, Mayhew CN, Rankin SA *et al.* Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. *Nature* 2011; **470**:105-U120.

4 Workman MJ, Mahe MM, Trisno S *et al.* Engineered human pluripotent-stem-cell-derived intestinal tissues with a functional enteric nervous system. *Nat Med* 2017; **23**:49-59.

5 Watson CL, Mahe MM, Munera J *et al.* An in vivo model of human small intestine using pluripotent stem cells. *Nature Medicine* 2014; **20**:1310-1314.