Migration and differentiation of transplanted enteric neural crest-derived cells in murine model of Hirschsprung's disease

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Abstract Stem cell therapy offers the potential of rebuilding the enteric nervous system (ENS) in the aganglionic bowel of patients with Hirschsprung's disease. P0-Cre/Floxed-EGFP mice in which neural crest-derived cells express EGFP were used to obtain ENS stem/progenitor cells. ENS stem/progenitor cells were transplanted into the bowel of $Ret^{-/-}$ mouse, an animal model of Hirschsprung's disease. Immunohistochemical analysis was performed to determine whether grafted cells gave rise to neurons in the recipient bowel. EGFP expressing neural crest-derived

cells accounted for 7.01 ± 2.52 % of total cells of gastrointestinal tract. ENS stem/progenitor cells were isolated using flow cytometry and expanded as neurosphere-like bodies (NLBs) in a serum-free culture condition. Some cells in NLBs expressed neural crest markers, p75 and Sox10 and neural stem/progenitor cells markers, Nestin and Musashi1. Multipotency of isolated ENS stem/progenitor cells was determined as they differentiated into neurons, glial cells, and myofibroblasts in culture. When co-cultured with explants of hindgut of $Ret^{-/-}$ mice, ENS stem/progenitor cells migrated into the aganglionic bowel and gave rise to neurons. ENS stem/progenitor cells used in this study appear to be clinically relevant donor cells in cell

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therapy to treat Hirschsprung's disease capable of colonizing the affected bowel and giving rise to neurons.

Keywords Hirschsprung's disease · Enteric nervous system · Stem cells · Cell therapy · Neural crest

Introduction

The enteric nervous system (ENS) is an extensive network of neurons and glial cells within the wall of the gastrointestinal tract. The ENS is critically important in regulating gut motility and other fundamental aspects of gut function (Furness 2012). As a result, enteric neuropathies, in which enteric neurons are either abnormal or congenitally absent, can cause significant morbidity. Hirschsprung's disease is characterized by absent ganglion cells in the distal bowel due to failure of neural crest-derived precursors to colonize during embryonic development. The aganglionic region is paralyzed, leading to colonic obstruction, and current treatment requires surgical resection of this aganglionic segment. While surgery is life-saving, over 50 % of children have persistent problems, including severe constipation or fecal incontinence (Catto-Smith et al. 2007; Ieiri et al. 2010). Cell-based therapy offers the potential to introduce new neurons into the aganglionic region to avoid the need for surgery and to improve outcomes.

Neural crest stem/progenitor cells can be isolated from embryonic and postnatal gut of mouse and humans (Natarajan et al. 1999; Bondurand et al. 2003;

Kruger et al. 2002; Almond et al. 2007; Metzger et al. 2009). These cells can give rise to neurons and glial cells when grafted into explants of chick, mouse or human gut (Mosher et al. 2007; Metzger et al. 2009; Lindley et al. 2008; Belkind-Gerson et al. 2013) or postnatal mouse gut in vivo (Hotta et al. 2013). Moreover, a recent human study has shown that enteric neuronal stem/progenitor cells can be obtained from mucosal biopsy specimens taken during routine endoscopy from children with Hirschsprung's disease (Metzger et al. 2009), demonstrating the potential for the use of patient-derived cells in the future for autologous cell replacement that circumvent ethical and immunological challenges.

To date, a number of investigators have reported different method to isolate and culture enteric neuronal stem/progenitor cells and most of the media contain serum or animal-derived products (Natarajan et al. 1999; Bondurand et al. 2003; Kruger et al. 2002; Almond et al. 2007). Growth of enteric neural crest-derived cells in medium lacking animal-derived products would eliminate the risk of contamination and reduce the possibility of a host immune response.

In this study, we tried to examine the potential ability of ENS stem/progenitor cells isolated from embryonic gut in mice, and our final goal is to apply results in mice study to the clinical transplantation therapy for the patient who is suffering from the aganglionic diseases.

Materials and methods

Animals

Timed pregnant mice were killed by cervical dislocation. All procedures were approved by the Keio University Animal Ethics Committee (Ethics ID: 09125). Transgenic mice expressing Cre recombinase under control of the P0 promoter (P0-Cre) (Yamauchi et al. 1999) were mated with EGFP reporter mice (CAG-CAT-EGFP) (Kawamoto et al. 2000) to obtain P0-Cre/Floxed-EGFP mice (P0-Cre/EGFP mice) in which neural crest-derived cells express EGFP within various tissues, including the gut (Nagoshi et al. 2008). Ret^{+/-} mice were kindly provided by Prof. Takahashi following permission from Prof. Costantini. The Ret^{+/-} mice were originally generated by Schuchardt et al. (1994) by targeted deletion of the c-Ret gene

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Table 1 Primary antisera

Antisera	Host	Concentration	Source of reference
α SMA	Mouse IgG2a	1:1,000	Sigma Aldrich Japan, Tokyo, Japan
β -III tubulin (Tuj1)	Mouse IgG2b	1:500	Sigma Aldrich Japan, Tokyo, Japan
GFAP	Rabbit IgG	1:500	Dako Japan, Tokyo, Japan
GFP	Rabbit IgG	1:500	MBL Co., LTD, Nagoya, Japan
GFP	Goat IgG	1:200	Santa Cruz Biotechnology, Dallas, TX, USA
Musashi 1	Rat IgG	1:500	Okano Laboratory (Kaneko et al. 2000)
Nestin	Mouse	1:500	Novus biologicals, Littleton, CO, USA
P75	Rabbit IgG	1:500	Chemicon Japan Co., LTD, Tokyo, Japan
Sox10	Goat IgG	1:200	R&D Systems, Inc., Minneapolis, MN, USA

Table 2 Secondary antisera

Species in which primary antisera were raised	Secondary antisera
Mouse IgG2a	Goat anti-mouse IgG2a Alexa 350 (1:1,000, Molecular Probes)
Mouse IgG2b	Goat anti-mouse IgG2b Alexa 488 (1:1,000, Molecular Probes)
Rabbit	Goat anti-rabbit Alexa 568 (1:1,000, Molecular Probes)
Rat	Goat anti-rat Alexa 555 (1:1,000, Molecular Probes)
Sheep/Goat	Donkey anti-sheep IgG Alexa 568 (1:1,000, Molecular Probes)
Nuclear staining was performed using Hoechst 33258 stain solution (Sigma Aldrich)	

(Schuchardt et al. 1994). $Ret^{-/-}$ mice die shortly after birth with no enteric neurons beyond the stomach (Schuchardt et al. 1994; Durbec et al. 1996).

Immunohistochemistry

For cryosections, entire gastrointestinal tract of post-natal mouse or neurosphere-like bodies (NLBs) were fixed in 4 % paraformaldehyde (PFA) overnight at 4 °C. Samples were embedded in cryomolds for sectioning at 14 μ m for bowel and at 10 μ m for NLBs. Catenary co-culture preparations

were fixed overnight in 4 % PFA at 4 °C. Samples were exposed to 0.1 % Triton X-100 for 30 min followed by processing for immunohistochemical staining using primary and secondary antisera (Tables 1 and 2). Anti-musashi-1 antibody was originally developed in our laboratory (Kaneko et al. 2000). We used Hoechst 33258 (DOJINDO LABORATORIES, Tokyo, Japan) reagent for nuclear staining. Preparations were viewed on a confocal microscope.

Isolation and expansion of enteric neural crest-derived cells from embryonic P0-Cre/EGFP mice

Termed pregnant female mice were sacrificed to obtain the P0-Cre/EGFP embryo at embryonic day 14.5 (E14.5). The entire gut from the stomach to the anus was dissected out and incubated in 0.25 % Trypsin–EDTA (Invitrogen-Life Technologies Japan, Osaka, Japan) for 1.5 h at 37 °C with gentle pipetting. The cell suspension was passed through a 70 μ m cell strainer (Falcon-Benedict Dickinson, Oxford, U.K.) and centrifuged at 800 $\times g$ for 5 min at 4 °C. The pellet was resuspended and EGFP positive cells were isolated by flow cytometry (MoFlo, Beckman Coulter K.K., Tokyo, Japan). Cells were seeded and plated at 5×10^4 cells/ml in DMEM/F12 medium containing 100 ng/ml recombinant human EGF (Pepro Tech, Rocky Hill, NJ, USA), 100 ng/ml human FGF-2 (Pepro Tech), 20 μ g/ml of B27 (Invitrogen-Life Technologies, Tokyo, Japan), and 2mM L-glutamine (Life Technologies, Tokyo, Japan) and cultured in an incubator at 37 °C, 5 % CO₂ for 7 days to allow NLBs formation (Nagoshi et al. 2008).

Flow Cytometry

Cell sorting and cell analysis were performed on a triple laser MoFlo (Beckman Coulter K.K., Tokyo, Japan) with Summit software as described previously (Matsuzaki et al., 2004). Briefly, 2 μ g/ml Propidium iodide (PI: Sigma-Aldrich Co., Tokyo Japan) was treated on 5 ml cell suspension with EGFP positive cell screening. PI fluorescence was measured after excitation at 488 nm with argon laser, and a live cell gate was defined that excluded the cells positive for PI. EGFP+ cells were identified by EGFP fluorescence.

Fig. 1 ENS stem/progenitor cells isolated from embryonic gut of P0-Cre/EGFP mice and expanded as neurosphere like bodies (NLBs). **a–c** Section through small intestine of post-natal P0-Cre/EGFP mice showed EGFP expressing cells in both myenteric plexus and submucosal plexus colocalizing with pan neuronal marker, PGP 9.5. *Scale bar:* 50 μ m. **d, e** NLBs derived from EGFP⁺ cells from the intestine of E14.5 P0-Cre/EGFP mice. EGFP⁺ cells isolated from E14.5 P0-Cre/EGFP mice gut grown in a floating culture for 7 days. A number of spheroid structures formed (**d**), resembling neurosphere like

bodies (NLBs) that are expressing EGFP (**e**). *Scale bar:* 100 μ m. **f–i:** Characterization of P0-Cre/EGFP mice gut-derived NLBs. NLBs contained cells expressing neural crest markers, p75 (**f**) and Sox 10 (**g**). A sub population of cells within the NLBs expressed neural stem cells markers, Nestin (**h**) and Musashi1 (**i**). *Scale bar:* 100 μ m. **j–l** In vitro characterization of NLBs derived from EGFP⁺ cells from the intestine of E14.5 mice. Following 7 days of culture on poly-D-lysine/laminin, a subpopulation of cells expressed the neuronal marker, Tuj1 (**j**), glial marker, GFAP, and α SMA (myofibroblasts). *Scale bar:* 50 μ m

Differentiation of NLBs in vitro

NLBs generated from the gut of E14.5 mice were transferred to glass bottom chamber slides coated with poly-D-lysine/laminin (Sigma-Aldrich, St. Louis, MO, USA/Invitrogen-Life Technologies Japan) and cultured in a medium containing 10 % fetal bovine serum (FBS; Equitech-Bio, Kerrville, TX, USA) for 4 days before being fixed in 4 % PFA and processed for immunohistochemistry.

Co-cultures of gut explants and NLBs

Explants of post-cecal hindgut were obtained from E12.5 mice. NLBs were apposed to the rostral end of explants of hindgut and co-cultured for 7 days as described previously (Hearn et al. 1999).

Results

Distribution of EGFP expressing enteric neural crest cells in the intestinal wall of P0-Cre/EGFP mice

To examine the distribution of EGFP expressing cells within the gastrointestinal tract of P0-Cre/EGFP mice, cryosectioned small intestines of post-natal P0-Cre/EGFP mice were examined. Clusters of EGFP expressing cells were located two separate layers within the bowel wall (Fig. 1a). Immunohistochemical staining with the pan-neuronal marker, PGP9.5 demonstrated co-localization of PGP9.5 positive cell clusters with EGFP expression (Fig. 1b, c), suggesting that neural crest-derived enteric neurons expressed EGFP in the myenteric and submucosal plexuses in P0-Cre/EGFP mice.

Isolation of proliferative neural crest-derived cells from embryonic gut of P0-Cre/EGFP mice

EGFP expressing enteric neural crest cells were isolated from dissociated gastrointestinal tracts of E14.5 P0-Cre/EGFP mice using flowcytometer. EGFP⁺ cells accounted for 7.01 ± 2.52 % of total cells ($n = 6$). When grown in medium containing 100 ng/ml EGF and 100 ng/ml FGF-2 in floating culture, spheroid structures resembling NLBs formed following 5–7 days culture (Fig. 1d). Most of the

NLBs were 50–100 μ m in diameter after 7 days in culture and all of the cells comprising NLBs were EGFP-positive (Fig. 1e). To characterize cells within the NLBs, frozen sections through NLBs were examined immunohistochemically using antibodies to markers of neural crest cells [p75 (Stemple and Anderson 1992) and Sox10 (Paratore et al. 2001)] and neural stem/progenitor cells [Nestin (Lendahl et al. 1990) and Musashi-1 (Sakakibara et al. 1996)]. A sub-population of cells within the NLBs was immunoreactive for p75 (Fig. 1f) and Sox10 (Fig. 1g). Moreover, the neural stem/progenitor cells markers, Nestin (Fig. 1h) and Musashi1 (Fig. 1i) were also expressed by a sub-population of cells in the NLBs. These data suggest that the EGFP⁺ cells isolated from the gut of P0-Cre/EGFP mice contained neural crest stem cells.

Differentiation of NLBs in vitro

To examine the ability of NLBs generated from the gut of P0-Cre/EGFP mice to give rise to neurons and other neural crest derivatives, NLBs were cultured on poly-D-lysine/laminin-coated chamber slides in medium containing 10 % FBS without EGF or FGF-2 for 7 days. Cells within the NLBs completely dispersed on the culture dishes and some extended neurites (data not shown). Immunohistochemical examination revealed that cells in the NLBs gave rise to neurons (Tuj1), glial cells (GFAP), and myofibroblasts (α SMA) (Fig. 1j–l). These findings suggested that isolated neural crest stem/progenitor cells from embryonic gut of P0-Cre/EGFP mice have an ability to give rise to appropriate derivatives in vitro. Taken together, our data show that EGFP expressing cells isolated from the gut of embryonic P0-Cre/EGFP mice were neural crest-derived cells capable of expanding as NLBs and giving rise to multiple cell types, including neurons. Therefore, we refer to these cells as ENS stem/progenitor cells.

Co-culture of NLBs and aganglionic hindgut of Ret^{-/-} mice

To examine whether the ENS stem/progenitor cells isolated from the gut of P0-Cre/EGFP mice can colonize explants of recipient gut, catenary co-cultures of NLBs and explants of hindgut from E12.5 Ret^{-/-} mice were set up. Most of the enteric neural crest cells

Fig. 2 Migration and differentiation of ENS stem/progenitor cells within the explants of aganglionic gut of $Ret^{-/-}$ mice. **a–c** P0-Cre/EGFP mice gut-derived NLBs were co-cultured with explants of aganglionic gut of E12.5 $Ret^{-/-}$ mice for 7 days. EGFP⁺ ENS stem/progenitor cells within the NLBs colonized aganglionic gut (**a**) with chain-like networks differentiating into neuronal marker PGP 9.5 expressing neurons (**b, c**). Scale bar:

200 μ m. **d–i** Grafted ENS stem/progenitor cells formed enteric ganglia-like cell clusters within the aganglionic recipient gut. **d–f** A number of cell clusters (arrows) that contain neurons were observed. Scale bar: 100 μ m. **g–i** High magnification of recipient gut showed that cell clusters-derived from grafted ENS stem/progenitor cells resembled enteric ganglia. Scale bar: 20 μ m

in $Ret^{-/-}$ mice die just after they enter the developing foregut, therefore $Ret^{-/-}$ mice do not have enteric neurons beyond the stomach (Schuchardt et al. 1994). After 7 days of co-culture, some EGFP⁺ cells from the NLBs had migrated into the recipient hindgut and formed chain-like networks along the gut (Fig. 2a) in a similar manner to normal ENS development (Young

et al. 2004). EGFP⁺ neurites were also observed. Furthermore, immunohistochemical staining showed that a sub-population of EGFP⁺ cells were immunoreactive to the pan-neuronal marker, PGP9.5 (Fig. 2b, c). Therefore, it is suggested that transplanted ENS stem/progenitor cells differentiated into neurons in the recipient gut. All of the PGP9.5⁺ neurons were

EGFP⁺, and thus were derived from the co-cultured NLBs, which confirms that the recipient hindgut of E12.5 Ret^{-/-} mice lacked enteric neurons (Fig. 2c).

A characteristic feature of the ENS is that the neurons are found in clusters, called ganglia. Higher magnification images of co-cultured recipient explants of Ret^{-/-} mice showed that the EGFP⁺ cells formed clusters (Fig. 2d–f). These cell clusters contained PGP9.5⁺ neurons (Fig. 2e, h) that co-expressed EGFP and were therefore derived from NLBs. These findings suggest that ENS stem/progenitor cells derived from the gut of embryonic P0-Cre/EGFP mice can migrate into explants of recipient embryonic bowel of Ret^{-/-} mice and give rise to clusters of neurons resembling ganglia.

Discussion

In the current study, enteric neural crest-derived cells isolated from the gut of P0-Cre/EGFP mouse formed NLBs that can colonize aganglionic gut of an animal model of Hirschsprung's disease and give rise to neurons.

P0-Cre/EGFP mouse used in this study is a unique and powerful tool for identifying neural crest-derived cells, since the expression of EGFP is specific and strong in various neural crest derivatives (Nagoshi et al. 2008; Yoshida et al. 2006; Tomita et al. 2005). In the P0-Cre/EGFP mice, cells were labeled by EGFP in which P0 promoter was once activated. The EGFP expression driven by CAG promoter persists beyond their proliferation and differentiation, because of the Cre-loxP reporter system. This is the first study to examine the distribution of EGFP⁺ cells in the gut of P0-Cre/EGFP mouse in detail. It was reported that P0 gene expression is activated transiently in a subpopulation of enteric neural crest-derived cells during the ENS development (Lee et al. 2001). Furthermore, this is consistent with immunohistochemical findings in the current study, demonstrating EGFP expressing cells were grouped into the two separate layers within the gut wall where cell bodies of enteric neurons are normally populated.

A number of different investigators have reported variety of markers expressed by enteric neuronal stem/progenitor cells (Almond et al. 2007; Metzger et al. 2009). Characterization of isolated ENS stem/progenitor cells from P0-Cre/EGFP mouse gut in the current

study shows they express markers of neural crest (p75 and Sox10) and neural stem/progenitor cells (Nestin and Musashi1). It has been reported that Nestin positive cells in enteric ganglia of mouse and human (Azan et al. 2011) form NLBs and give rise to neurons and glia in culture (Suarez-Rodriguez and Belkind-Gerson 2004; Belkind-Gerson et al. 2013). Musashi1, a well-known marker for stem cells in nervous system (Sakakibara et al. 1996; Okano et al. 2002) and intestine (Potten et al. 2003), plays a key role in maintaining undifferentiated status of neural stem cells (Okano et al. 2002). Sox10 and endothelin 3 have been proposed to play a similar role in the ENS (Bondurand et al. 2006), however, the role of Musashi1 in enteric neural crest-derived cells is still unclear.

To date, several papers have reported protocols to isolate and culture enteric neuronal precursors (Bondurand et al. 2003; Almond et al. 2007; Metzger et al. 2009). Most of the protocols used a medium containing serum or undisclosed animal-derived supplements, such as fetal bovine serum or chicken embryo extract. Serum contains variable and undefined amounts of soluble growth and differentiation factors and animal-derived proteins may trigger immunological reactions when placed in the human body. Hence the use of these supplements may hamper analysis and cause negative effects. In this study, we successfully cultured enteric neuronal stem/progenitor cells in a serum-free medium. This serum-free medium has been shown to be useful for culturing other stem cells, including CNS neural stem cells (Reynolds and Weiss 1992; Sawamoto et al. 2001) and neural crest stem cells (Nagoshi et al. 2008).

In our current study, the ability of migration of transplanted ENS precursors was very limited even when grafted into aneural gut. This is consistent with findings from previous reports (Natarajan et al. 1999; Lindley et al. 2008; Metzger et al. 2009; Hotta et al. 2013) and again raised a question how to bridge the gap between limited migratory capacity of grafted cells and reasonably long distance for them to travel covering the aganglionic gut segments. Recently, several investigators from leading laboratories have shown that neurogenesis occurs in ENS of adult rodents ex vivo (Becker et al. 2013) and in vivo (Liu et al. 2009; Joseph et al. 2011; Laranjeira et al. 2011). Liu et al. (2009) firstly demonstrated enteric neurogenesis in adult mice induced by stimulation of 5-HT₄

signaling and the more recent study utilizing genetic fate mapping has shown that Sox10-expressing common precursors contribute to neurogenesis in response to ENS injury (Laranjeira et al. 2011). These findings suggested the possibility to restore gut motility of patients with enteric neuropathies by enhancing endogenous enteric neurogenesis (Becker et al. 2013) potentially combined with introduction of engineered neuronal precursors (Sun et al. 2013). However, further studies will be required to understand the mechanism underlying enteric neurogenesis, which also has been shown to occur under extremely limited condition (Joseph et al. 2011).

Finally, we confirmed ENS stem/progenitor cells isolated from P0-Cre/EGFP mouse have an ability to colonize the aganglionic colon of Ret^{-/-} mouse at least in vitro. Moreover, some cells formed enteric ganglia-like cell clusters, giving rise to neurons. Formation of group of neuronal cell bodies, called ganglia is a characteristic feature of ENS. β 1-integrin and BMP-signaling have been implicated in ganglion formation during development of the ENS as depletion of β 1-integrin or perturbations in BMP-signaling result in a failure of enteric neural crest cells to cluster properly and form normal shaped and sized ganglia (Goldstein et al. 2005; Fu et al. 2006; Breau et al. 2006). Although it is implicated that even aganglionic gut may contain environmental cue or molecules supporting transplanted enteric neuronal stem/progenitor cells to form ganglia-like cell clusters (Hotta et al. 2013), its underlying mechanism is not clearly understood.

Collectively, in the current study, we showed that ENS stem/progenitor cells isolated from embryonic gut of P0-Cre/EGFP mice can be expanded as NLBs in serum-free culture medium and they can colonize aganglionic gut of E12.5 Ret^{-/-} mouse, an animal model of Hirschsprung's disease. Furthermore, ENS stem/progenitor cells formed cell clusters, resembling enteric ganglia within the explants of recipient gut and finally gave rise to enteric neurons. They are significant findings for the potential application of cell-based therapy to treat Hirschsprung's disease in terms of establishing a platform to develop safer culture method of donor cells and to investigate the behavior of grafted cells following transplantation into aganglionic recipient colon.

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