Journal of Pediatric Surgery 57 (2022) 61–68

Contents lists available at ScienceDirect

Journal of Pediatric Surgery

journal homepage: www.elsevier.com/locate/jpedsurg.org

Review Article

Opportunities for novel diagnostic and cell-based therapies for Hirschsprung disease

Weikang Pan[a,b], Allan M. Goldstein[a], Ryo Hotta[a,*]

[a] Department of Pediatric Surgery, Massachusetts General Hospital, Harvard Medical School, 55 Fruit Street, 185 Cambridge St, CPZN 6-215, Boston, MA 02114, USA

[b] Department of Pediatric Surgery, The Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi 710004, China

ARTICLE INFO

Article history:
Received 7 July 2021
Revised 17 October 2021
Accepted 28 October 2021

Keywords:
Hirschsprung disease
Enteric nervous system
Neuronal stem cells
Cell therapy

ABSTRACT

Despite significant progress in our understanding of the etiology and pathophysiology of Hirschsprung disease (HSCR), early and accurate diagnosis and operative management can be challenging. Moreover, long-term morbidity following surgery, including fecal incontinence, constipation, and Hirschsprung-associated enterocolitis (HAEC), remains problematic. Recent advances applying state-of-the art imaging for visualization of the enteric nervous system and utilizing neuronal stem cells to replace the missing enteric neurons and glial cells offer the possibility of a promising new future for patients with HSCR. In this review, we summarize recent research advances that may one day offer novel approaches for the diagnosis and management of this disease.

1. Introduction

Hirschsprung disease (HSCR) is a developmental disorder of the enteric nervous system (ENS), characterized by the absence of enteric neurons and glial cells along variable length of the distal intestine. Current treatment of HSCR, especially in cases of extensive aganglionosis, remains inadequate. Treatments are limited to bowel resection for the majority of cases or diversion and parenteral nutrition for those with aganglionosis extending to the proximal small intestine. Surgical resection of the aganglionic segment has been the mainstay of treatment since Orvar Swenson's original description in 1948 [1] that revolutionized the management and survival of children with HSCR, a disease that had been universally fatal prior to that time. Mortality has been below 6% since the 1980s and is usually due to Hirschsprung-associated enterocolitis (HAEC) or iatrogenic complications of central venous catheter-related sepsis or parenteral nutrition-related liver failure, particularly in long-segment HSCR [2,3]. However, postoperative morbidity following pull-through surgery for HSCR remains a major problem. Recent studies report that HSCR patients frequently have impaired bowel function after pull-through surgery, with 42% of children reporting a clinically significant reduction in overall quality of life [4]. The frequency of defecation often remains persistently elevated into adulthood in up to 50% of cases [5]. This morbidity may be attributed to multiple causes, including abnormalities of, or operative injury to, the anal sphincters and/or pelvic innervation, residual dysganglionosis or underlying colonic dysmotility, loss of the rectum as part of the pull-through operation, or functional constipation associated with the repeated trauma of anorectal dilations, irrigations, and enemas. The current results highlight the need for improved therapies for HSCR. In this review, we provide an overview of recent advances in clinical diagnosis and the status of regenerative cell-based approaches to treating HSCR.

2. Clinical diagnosis

2.1. Advances in diagnostic histopathology

At present, the ability to identify ganglion cells by histopathological evaluation of rectal biopsies is primarily dependent on routine hematoxylin and eosin (H&E) stain and a pathologist's experience. Several studies have highlighted the advantages of combining H&E histochemistry with other immunohistochemistry (IHC) markers, leading to improved diagnostic accuracy [6,7]. Acetylcholinesterase (AChE) histochemistry has been used for many years to diagnose HSCR. However, it requires special tissue handling and cannot be performed on formalin-fixed, paraffin-embedded tissue. Calretinin IHC has been shown to have high specificity and sensitivity for diagnosing HSCR [7]. It is a very reliable adjunct to H&E staining for the evaluation of rectal biopsies [8] and has replaced the use of AChE staining at many centers. Another po-

Abbreviations: HSCR, Hirschsprung disease; ESCs, embryonic stem cells; iPSCs, induced pluripotent stem cells; CNS-NSCs, central neural system-neural stem cells; ENCDCs, enteric neural crest-derived cells; SKPs, skin-derived precursors; hDPSCs, human dental pulp stem cells.

* Corresponding author.
E-mail address: rhotta@mgh.harvard.edu (R. Hotta).

https://doi.org/10.1016/j.jpedsurg.2021.10.049
0022-3468/

tential alternative to AChE is staining for the choline transporter (ChT), which is responsible for transporting choline into cholinergic neurons where it is combined with acetyl CoA by choline acetyltransferase (ChAT) to make the excitatory neurotransmitter, acetylcholine (ACh). Kapur et al. found that abnormal mucosal ChT staining was present through the entire length of the aganglionic segment and into the transition zone, where hypertrophic submucosal nerves were observed [6]. The result is important for biopsies from patients with very short-segment HSCR since calretinin staining can be present in some of these cases, leading to false-negative results [9]. Galazka et al. recently studied a panel of antibodies and reported that the use of antibodies against CD56 (neural cell adhesion molecule, NCAM) and S-100 (glial marker) yielded a high sensitivity and specificity for the diagnosis [10]. However, the utility of immunohistochemistry is limited in the intra-operative setting, when timely identification of the aganglionic, ganglionic, and transition zones is essential.

2.2. Advanced imaging techniques for diagnosis

Although the typical clinical presentation of HSCR is that of a neonate who fails to pass meconium within 48 h of life [11], the presentation can vary. While the majority present during infancy, some develop severe constipation after the neonatal period. In about 10% of cases, the initial presentation is with diarrhea due to HAEC, often delaying the diagnosis [12]. Patients for whom there is a high index of suspicion for HSCR typically undergo contrast enema or anorectal manometry. These are less invasive for initial screening, but their specificity and positive predictive value are significantly lower [13,14] than rectal suction biopsy (RSB), the gold standard for the diagnosis with 95–97% sensitivity and 99% specificity [15]. However, challenges exist with RSB, such as sampling bias caused by the analysis of small specimens [16]. In addition, inadequate RSB and false-negative results are higher in older children due to the thickness of the rectal mucosa, requiring open transanal rectal biopsy in these patients and general anesthesia. A recent systematic analysis showed that RSB-related complications, such as bleeding and perforation, are more frequent in newborns and infants than in older children [15]. These studies indicate the need for more accurate and less invasive techniques for improving the diagnosis of HSCR.

Over the last decade, several innovative studies have reported successful visualization of the myenteric plexus without tissue processing for immunohistochemistry, which would be beneficial for real-time, non-invasive diagnosis and for determination of the appropriate level for surgical transection intra-operatively. Frykman et al. for example, showed that spectral imaging could distinguish normal *versus* aganglionic bowel with high sensitivity and specificity in mice with HSCR [17]. Dubois et al. developed full-field optical coherence microscopy (FFOCM) based on the technology used for optical coherence tomography (OCT), which is widely used in biomedical imaging, including endoscopy [18]. The low resolution of OCT was improved by adding the high transverse resolution of confocal microscopy. This wide-field, high-resolution form of OCT can acquire <1 μm resolution images of deep tissue without requiring any contrast dye [19]. Coron et al. demonstrated the ability of FFOCM to visualize the myenteric plexus in surgically resected human gut as well as mouse intestine [20]. FFOCM was able to distinguish the ganglionic from aganglionic segments in mice with HSCR, using either fresh or fixed specimens [20]. However, further validation of this technique using a larger number of human specimens and optimization for its application *in vivo* are needed.

Confocal laser endomicroscopy (CLE) is another potential tool for visualizing the human myenteric plexus. An initial report using this technology showed successful visualization of the ENS during endoscopy in patients with colon cancer, although endoscopic removal of the mucosa and administration of neuronal dye were necessary [21]. Serosal application of the probe was used on surgically resected human intestine to test the feasibility of this imaging modality [22] and showed successful visualization of myenteric ganglia with 88.4% accuracy [23]. Although some technical challenges, such as adjusting the focal plane of CLE to the thin myenteric layer, must be overcome before clinical application, these findings raise the possibility for using minimally invasive techniques to diagnose HSCR and to identify the transition zone either endoscopically or laparoscopically. A recent pilot study using ultra-high frequency (UHF) ultrasound demonstrated successful visualization of key structures of the colorectal wall by serosal imaging of surgically resected ganglionic and aganglionic segments of bowel from children with HSCR [24]. While this study used *ex vivo* specimens, it lays the foundation for potentially using UHF ultrasound for *in vivo* diagnosis and as an aid in determining the resection margin during pull-through surgery or the proper location of a leveling ostomy. Developing advanced imaging technologies for visualization of the ENS would not only permit safe, accurate, and minimally invasive circumferential evaluation of the intestine, but would also allow large studies of healthy populations at multiple ages to determine the normal number, density, and structure of enteric ganglia. This would provide much needed normative data about the structure of the normal human ENS at different ages and in different regions of the bowel, and thereby improve our ability to define the transition zone and to diagnose more subtle dysganglionoses than HSCR.

A limitation of current diagnostic techniques is that they are only intended to identify the presence or absence of ganglion cells, whereas the pathology of HSCR is more complex. Several studies have shown altered ratios of inhibitory and excitatory enteric neurons at the proximal resection margin, with evidence suggesting that this may contribute to postoperative morbidity [25]. Furthermore, diagnosing hypoganglionosis in the transition zone remains subjective and this may lead to failure to remove completely this segment at the time of the pull-through. Therefore, the simple presence of ganglion cells may not be sufficient to determine the optimal resection margin. Future diagnostic modalities will ideally assess neuronal density and subtype composition as an intraoperative determinant of resection level and as a predictor of clinical outcome.

2.3. Role of genetic diagnosis

The role of genetic testing for the prenatal diagnosis of HSCR remains unclear. The disease is non-Mendelian with variable phenotypic expression and incomplete penetrance, reflecting complex interactions among multiple genetic loci and epigenetic/environmental factors and making diagnosis based on genetics alone unlikely. Genetics can, however, provide risk stratification and identify high-risk newborns, which can alert clinicians and help ensure early clinical diagnosis. While absolute risk assessment may be difficult, certain high-risk factors have been identified. For example, a child of a parent with long-segment HSCR has about a 20% risk of disease [11]. Badner et al. determined risk in siblings based on the sex of the proband and of the future child and the length of aganglionosis [26]. The highest risk was 33% and occurred in male siblings of affected females with long-segment disease. The majority of HSCR cases, however, are sporadic and not associated with a family history. Tilghman et al. recently showed that HSCR stems from common noncoding variants, rare coding sequence variants, and copy number variants affecting genes involved in ENS development, especially in the RET protein regulatory network [27]. One of these three genetic features was present in 72% of HSCR patients. Interestingly, individuals with none of these causal factors have a 1 in 18,800 risk of disease, while those

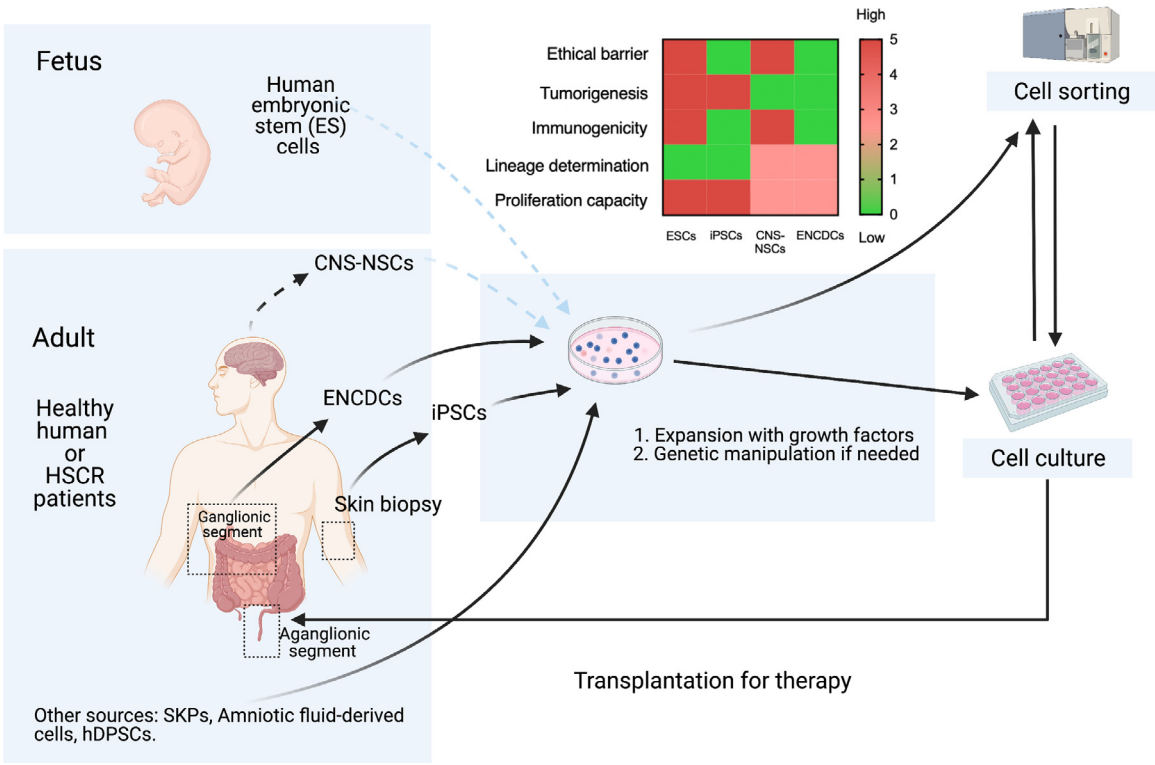

Fig. 1. *Strategy for cell therapy for enteric neural disorders*. Donor cells can be harvested from fetal or postnatal tissue, with potential cell sources including ESCs, iPSCs, CNS-NSCs, ENCDCs, or other novel sources such as SKPs, amniotic fluid-derived stem cells, and hDPSCs. Cells are expanded in culture, with the option of performing genetic modification and/or cell sorting prior to transplantation into the affected segment of the GI tract. Each cell source has potential ethical barriers, tumor risk, immunogenicity, lineage potential, and proliferation capacity, as summarized above. For example, ESCs have the advantage of pluripotency and a robust proliferation capacity but present ethical, tumorigenic, and immunogenicity challenges. iPSCs show similar tumor risk and proliferation capacity as ESCs, but if derived autologously, the immunogenic risk is avoided. CNS-NSCs have potential advantages but cannot be derived autologously, whereas autologous ENCDCs have high lineage determination given their neuronal commitment, lower proliferation capacity than ESCs and iPSCs, and low tumorigenicity and immunogenicity.

with all three factors have a 1 in 120 risk. While genetics cannot yet provide a definitive prenatal diagnosis, and will not replace rectal biopsy postnatally, it can provide valuable risk stratification that can help raise a clinician's level of suspicion and their decision on whether to pursue definitive testing. A child with high genetic and familial risk, even in the absence of clear clinical symptoms as a neonate, may thus merit a rectal biopsy to avoid a delay in diagnosis and the risks inherent in that.

3. Stem cell therapy for HSCR

Over the past twenty years, significant progress has been made in the pursuit of cell replacement therapies for several human diseases, including neurodegenerative disorders [28]. Growing evidence has shown the feasibility of delivering enteric neural progenitor cells to ameliorate GI dysmotility in rodent models of enteric neuropathies [29,30], including HSCR [31]. In this section, we discuss recent progress with stem cell-based therapy for the treatment of HSCR and provide our perspective on how this therapy can be utilized in the management of this disease (Fig. 1).

3.1. Sources of stem cells

3.1.1. Enteric neural crest-derived cells (ENCDCs)

The cells that comprise the ENS are derived from the neural crest and are referred to as enteric neural crest-derived cells (ENCDCs). During development, these cells are instructed to migrate, proliferate, and differentiate into neurons and glia along the entire GI tract in response to multiple signaling pathways [32]. ENCDCs isolated from laboratory animals have been widely accepted as a useful tool to study the development of the ENS and as a potential cell source for regenerative therapy for HSCR. In one of the earliest studies on cell-based therapy for this disease, Natarajan et al. demonstrated in 1999 the potential use of embryonic mouse gut-derived ENCDCs for the treatment of HSCR by transplanting them to embryonic aganglionic hindgut explants obtained from *Ret*$^{-/-}$ mice, a model of HSCR [33]. Subsequently, several investigators successfully isolated ENCDCs from embryonic and postnatal rodent gut [34–36] and demonstrated their survival up to 24 months following transplantation in mouse colon *in vivo* [37]. ENCDCs give rise to functioning neurons [38,39] that integrate into the neuronal circuitry of the host ENS *in vivo* [40]. Importantly, ENCDC transplantation was recently shown to ameliorate the decreased colonic motility seen in neuronal nitric oxide synthase (nNOS)-null mice four weeks after surgery [29].

ENCDCs can be isolated from the ganglionated human intestine [41–44], mucosal biopsy samples [45], and even from aganglionic distal colon resected from patients with HSCR [46]. These cells can migrate and differentiate into neurons and glial cells following transplantation to ganglionated mouse colon *in vivo* [47]. Although these results support the feasibility of isolating donor cells from human patients with HSCR, the reduced capacity of postnatal ENCDCs to proliferate and differentiate has been a limiting factor [36]. Therefore, manipulation of donor HSCR-derived ENCDCs prior to transplantation is required to optimize their therapeutic potential and this can be achieved by growing them in appropriate culture conditions supplemented with growth factors that maximize the number of undifferentiated progenitor cells [48].

3.1.2. Pluripotent stem cells (PSCs)

Pluripotent stem cells (PSCs), including human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have a ca-

pacity to self-renew indefinitely and to develop into the three germ layers of the embryo. These characteristics grant them an enormous advantage in the field of regenerative medicine. Several protocols have been established for the efficient derivation of specialized neural cell types [49,50], including enteric neurons via induction of a neural crest lineage from PSCs [51,52]. Fattahi et al. described an efficient strategy to derive ENS lineages from human PSCs (hPSCs) via induction to neural crest cells and purification following specification [31]. Subsequent validation of these cells demonstrated functional integration into neuromuscular circuitry with the expression of a wide range of neurochemical subtypes *in vitro*. Importantly, these cells were able to rescue disease-related mortality in HSCR mice ($EDNRB^{s-l/s-l}$) following transplantation of PSC-derived ENS progenitor cells to the colon *in vivo* [31]. This work had a significant impact in demonstrating the potential utility of hPSCs for the treatment of enteric neuropathies, although it remains unclear how the transplanted cells were able to improve survival in these mice, whether it was by improving gut motility, reducing HAEC risk, or another mechamism.

A beneficial characteristic of iPSCs is that they can be generated from terminally differentiated cell types and therefore can be autologously derived. This is advantageous because they could bypass the ethical and immunological considerations associated with embryonic stem cells. Furthermore, genetic mutations in patient-derived stem cells can be corrected. Lai et al. established iPSCs from patients with HSCR due to a mutation in the RET gene [53]. Enteric neural crest cells generated from these iPSCs exhibited defects in migration and neuronal differentiation *in vitro*, but these were functionally restored with CRISPR/Cas9 gene-editing technology. In another recent study, Li et al. showed that hPSCs could efficiently generate functional enteric-like neurons [54]. When transplanted into chicken, rodent, or human aganglionic gut tissues, hPSCs-derived neural crest cells were able to migrate and undergo neural differentiation. These results indicate that hPSCs represent a potential autologous source for cell-based therapy. However, long-term outcomes, particularly the risks of tumor formation and graft-related immune effects after transplant, need to be addressed.

3.1.3. Central nervous system-derived neural stem cells (CNS-NSCs)

In 1992, Reynolds and Weiss succeeded for the first time in isolating neural stem cells from rodent brain and expanding them in culture [55]. This discovery provided strong evidence that adult tissue contains undifferentiated cells that may contribute to replenishing dying or damaged cells, even in the brain, an organ long considered to have limited or no regenerative ability. CNS-NSCs were among the first cell types tested for ENS therapy. Micci et al. showed that these cells predominantly gave rise to neuronal nNOS expressing neurons, which resulted in electric field stimulation-induced gut relaxation [30]. When CNS-NSCs were transplanted to the pylorus of the nNOS-null mouse, a model of gastroparesis, they led to an improvement in gastric emptying one week later. These authors also demonstrated improved cell survival following transplantation by inhibiting apoptosis in the donor cells [30]. However, very little progress has been achieved using CNS-NSC to treat GI disorders over the last 10 years, largely because of their limited potential for clinical application. To generate CNS-NSCs to treat human diseases, brain tissue would need to be harvested from human embryos, raising ethical concerns. Moreover, since CNS-NSCs cannot be autologously derived, immunological challenges would be unavoidable.

3.1.4. Other cell sources

It is well known that cells from the neural crest migrate extensively during development and widely distribute throughout the body. The skin contains progenitor cells derived from neural crest and possessing neurogenic potential *in vitro*. These skin-derived precursors (SKPs) have been successfully isolated from rodents [56], swine [57], and humans [58]. A recent *in vivo* study reported human SKPs successfully engrafting one week following transplantation to the colon of swine *in vivo*. Interestingly, autologously derived SKPs have been transplanted to aganglionic porcine bowel generated by treatment with the detergent, benzalkonium chloride (BAC), *in vivo*. Thomas et al. observed neuroglial differentiation of autologous SKPs within the aganglionic gut environment seven days after transplantation [57]. This is the first demonstration to suggest the feasibility of autologous cell therapy to treat aganglionosis *in vivo*, but further investigation is warranted to address cell survival and differentiation in the long-term.

The ability of amniotic fluid-derived stem cells to differentiate into various lineages has made them powerful tools for regenerative medicine. Zhou and Besner demonstrated that amniotic fluid-derived cells could be induced into NSCs *in vitro* [59]. They also observed that colonic motility of Ednrb KO mice was improved one week after transplantation of these cells, although cell survival beyond one week within the aganglionic colon *in vivo* was not observed.

During the last decade, dental pulp stem cells (DPSCs) have received extensive attention in regenerative medicine. DPSCs reside within the perivascular niche of the dental pulp. They originate from neural crest cells [60] and possess mesenchymal stem cell properties [61]. DPSCs have been isolated from mouse and human and possess the ability to differentiate into various neural cell types [62,63]. Arthur et al. showed that exposure of DPSCs to the appropriate environmental cues is required for them to differentiate into functionally active neurons [64]. These cells, for example, acquire a neuronal immunophenotype following transplantation into the brain of chicken embryos. Human DPSCs have been transplanted into the rodent model of spinal cord injury and showed marked recovery of hindlimb locomotor function [65] by inhibiting multiple axonal growth inhibitors via paracrine mechanisms. These studies suggest that neural crest-derived DPSCs can be a potential cell source for cell-based therapy to treat HSCR. However, there are no published studies yet showing DPSCs can give rise to enteric neurons following transplantation to the gut wall or their ability to elicit functional improvement in GI motility.

3.2. Challenges to overcome before clinical application

As discussed above, several promising studies show significant advances in the feasibility and therapeutic potential of cell-based approaches to treat HSCR. However, there are several unresolved challenges that need to be addressed before clinical application. These critical hurdles, and approaches to addressing them, are discussed below.

3.2.1. Is administration of enteric neural progenitor cells sufficient to treat HSCR?

Treating HSCR by cell replacement relies on the idea that HSCR is a developmental disorder due to a deficit of neural crest-derived cells without significant alteration in other cell types. However, the evidence suggests that abnormalities in HSCR extend beyond the ENS, including interstitial cells of Cajal (ICCs) [66], smooth muscle [67], epithelial and immune function [68,69], and altered microbiome [70]. Although the underlying mechanisms are not fully understood, these studies raise the possibility that delivering neural progenitor cells to replace the missing ENS may not be enough to restore gut function fully in HSCR. However, two recent studies showed that transplanting ENS progenitors restored architectural changes in the aganglionic colon [71] and also reversed the reduction in the number of ICCs seen in nNOS-deficient mice [29]. These findings demonstrate the interplay between cells of the ENS

and neighboring cells in the gut, but whether any of the HSCR-associated abnormalities outside of the ENS will remain problematic following ENCDC transplantation remains unknown. For example, if epithelial and immune function are not normalized after cell therapy, the patient may remain susceptible to HAEC. Current studies need to examine the effects of cell transplantation on these other aspects of gut function in addition to intestinal motility.

3.2.2. Will autologous ENCDCs from HSCR patients work?

Autologous ENCDCs represent a promising cell source for HSCR patients. However, it remains unclear whether these cells need to be genetically modified to correct their underlying mutation prior to transplantation. Characterizing their neurogenic, mitogenic, and migratory potential is essential to develop autologous cell therapy as a viable approach. We and others have shown that ENCDCs isolated from Ednrb-deficient mice and therefore lacking the *Ednrb* gene possess an equivalent ability to migrate and differentiate into neurons as ENCDCs isolated from wild-type mice [72,73]. However, as mentioned above, Lai et al. showed defects in migration and neuronal differentiation in ENCDCs from iPSCs established from patients with HSCR associated with *RET* mutations, and were able to rescue these deficits *in vitro* using CRISPR/Cas9 to correct the mutation [53]. While correcting an underlying mutation prior to isolating cells for transplant is appealing, the fact is that the majority of HSCR cases are not associated with coding sequence mutations, but rather with noncoding variants, copy number variation, and other genetic and/or environmental factors, limiting the potential utility of gene editing. Moreover, most cases of HSCR are short-segment, meaning that the neural crest cells were able to form an ENS in > 95% of the length of the bowel despite an underlying genetic abnormality. Thus, following massive expansion of autologous-derived cells *in vitro*, we believe they would be capable of forming an ENS in the residual aganglionic segment.

3.2.3. Is the postnatal aganglionic gut environment permissive for transplanted neural progenitors?

During development, neural crest cells migrate in an embryonic mesenchymal environment that promotes their development by secreting neurotrophic factors, such as Gdnf [74], and providing a supportive extracellular matrix [75]. Therefore, HSCR gut mesenchyme may lack important factors required for the survival and maintenance of enteric neural progenitors. Furthermore, the postnatal gut environment is markedly distinct from its embryonic state, raising concern that the postnatal aganglionic environment could present a barrier to transplanted progenitor cells [76]. Despite this, exogenous ENCDCs have been shown to survive up to 24 months and to differentiate into neurons following transplantation into postnatal ganglionic mouse colon, where they proliferate, migrate extensively, and differentiate into cells with the neurochemical, morphological, and electrophysiological characteristics of enteric neurons, including making synaptic connections [29,37,38,40]. However, it remains unknown if ENS progenitors can similarly engraft and mature in the postnatal aganglionic environment *in vivo*. This is largely due to the lack of an appropriate animal model of colonic aganglionosis, especially since transgenic aganglionic mice are lethal early in life, preventing examination of long-term outcomes after cell therapy. Recently, we developed a novel model of aganglionosis in which enteric neural crest-derived cells express diphtheria toxin (DT) receptor [71]. Local injection of DT into the colonic wall leads to focal aganglionosis that persists for several months and is non-lethal, allowing long-term analysis of the fate of ENCDCs transplanted into the aganglionic colon segment. ENCDC transplantation into this experimentally induced aganglionic colon led to neuroglial engraftment, migration, and differentiation, as well as restoration of normal structure to the colonic epithelium and muscle layers, suggesting that transplanted progenitors can survive and function in the aganglionic environment.

3.2.4. Can sufficient numbers of enteric neural progenitor cells be generated for transplantation?

It is known that tissue-derived adult stem cells are a rare cell population, which creates a challenge if autologous gut-derived ENCDCs are the donor cell of choice. Fox example, Kruger et al. observed that the frequency of enteric neural crest stem cells in the gut wall declines with the age of animals, dropping from 0.5–1% in young postnatal (P5–22) mice to only 0.2% in adult (P65–110) mice [36]. Similar observations were made using *Wnt1::Cre;R26R-YFP* mice, in which neural crest-derived cells express fluorescent protein YFP, showing that only 1.2% of cells obtained from the intestinal muscular layer are YFP^+ in postnatal mice (postnatal day 8), whereas the frequency in the embryonic gut (embryonic days 12–15) is over 7% [77]. Furthermore, many investigators have shown that postnatal gut derived ENCDCs grow more slowly than embryonic ENCCs, and their potential to differentiate and migrate decrease significantly [78]. These characteristics raise concern regarding whether adequate numbers of donor progenitor cells can be obtained from postnatal gut tissue.

To overcome this concern, several studies have been carried out to optimize culture conditions to maximize the number of ENCDCs. McKeown et al. showed that GDNF treatment resulted in a 12-fold increase in the number of ENCDCs grown in culture, and that low oxygen conditions stimulate proliferation of neural crest cells, resulting in $a >$ 2-fold increase in the proportion of $p75^+$ cells in postnatal mouse gut-derived neurospheres [79,80]. Similar beneficial effects have also been reported with addition of other factors, including 5-HT_4 [81], retinoic acid [82], endothelin-3 [83], glycogen synthase kinase 3 inhibitor [41], and granulocyte-colony-stimulating factor (GCSF) [84]. Multiple signaling pathways are involved in the expansion of the ENCDCs in culture, and synergistic effects among them have been shown [83,85]. We recently observed that the population of non-neural crest-derived cells present within the gut environment secrete factors that support ENCDCs in culture [86], further highlighting the need to characterize the signals that promote the growth of ENCDCs and leverage these for *in vitro* expansion of these cells.

When it comes to generating sufficient cell numbers, PSCs have a significant advantage since they proliferate indefinitely. However, PSCs cannot be transplanted in their pluripotent state due to the risk of tumor formation. Instead, PSCs are induced to the specific cell type needed before transplantation, such as enteric neurons via neural crest induction. Although protocols have been optimized for their induction and differentiation, PSCs still require a complex set of sequential exposures to specific signaling molecules in a time-sensitive manner that takes more than a month before generating enteric neurons [31,87,88]. Recent studies used RNA sequencing to understand the transcriptional regulation during commitment of PSCs to specific cell types via common progenitor cells. These data sets could be an important platform for optimizing the proliferation and differentiation potential of the neural crest lineage by modifying mitogenic and differentiation cues [89,90].

3.3. Other potential approaches for treating neurointestinal diseases

3.3.1. Tissue-engineered small intestine (TESI)

As discussed above, there have been a number of published articles demonstrating the potential of cell-based therapy to treat HSCR, but there remain challenges to overcome. In this section, we discuss alternative regenerative approaches that may circumvent these hurdles.

Tissue-engineered small intestine (TESI) was first generated from organoid units derived from dissociated rat small intestines

containing epithelial and mesenchymal cell types [91]. Organoid units were seeded on a tubular biodegradable scaffold, then grown under the renal capsule or implanted into the omentum of a host animal to form TESI for 4 to 6 weeks [92]. The resulting engineered intestine was morphologically similar to the native intestine, with muscle layers containing all key differentiated cell types, including enterocytes, enteroendocrine cells, Paneth cells, goblet cells, neurons, and glia [93]. This work has been extended to large animals and yielded successful engraftment in an autologous porcine model [94] and a xenograft model using human postnatal intestine derived TESI [95]. In rats, transplantation of TESI helped maintain the body weight of host animals and improved serum level of vitamin B12 following massive small bowel resection [92], suggesting that the regenerated epithelial components can absorb nutrients. More recent success in intestinal regeneration using organoid units have demonstrated functional recapitulation of contractility [96] and immune function [97]. Although TESI offers the possibility of generating autologous tissue of normal architecture and function to treat intestinal disorders, including HSCR, the process still needs optimization to generate adequate amounts of functional tissue rather than only small amounts of closed, cystic structures.

3.3.2. Human intestinal organoids (HIO)

Three-dimensional intestinal organoids have been generated from mouse [98,99] and human PSCs [88,100,101]. Early studies showed that, in the proper conditions, PSCs spontaneously differentiate and develop into intestinal organoids containing most of the cell types of the intestine except for the ENS [99,100]. Subsequent efforts led to the introduction of ENS components into human intestinal organoids (HIOs) by co-culturing HIOs with ENCDCs derived from PSCs [31] before their implantation under the renal capsule [101].

Workman et al. showed that ENS derived from PSCs via induction of enteric neural crest cells was able to integrate into the intestinal smooth muscle and promote nitric oxide-dependent relaxation, suggesting the formation of a functional neural circuitry [88]. Furthermore, Schlieve et al. demonstrated various neuronal subtypes and glia forming in the submucosal and myenteric layers of HIO-derived TESI [101]. Additionally, the contractility of HIO-TESI was shown under specific conditions. HIOs may represent a promising mechanism to replace abnormal intestinal tissue with a mature and functional tissue-engineered gut. Furthermore, HIOs provide a unique and valuable *in vitro* platform for drug screening to study the development of the ENS and the pathogenesis of HSCR. However, the small size and cystic shape of HIOs are ongoing challenges in need of optimization.

4. Conclusion and perspective

HSCR continues to represent a significant clinical challenge to neonatologists, geneticists, surgeons, and gastroenterologists. While we have made significant progress in its diagnosis and management over the past several decades, opportunities exist to improve the long-term outcomes of children with this disease. First, early and accurate diagnosis is essential to avoid missing cases that present later with failure to thrive, profound obstipation, or life-threatening enterocolitis. Leveraging improvements in histopathologic techniques, advancements in genetics, and new developments in optical imaging will allow us to achieve a prompt and accurate diagnosis in the vast majority of cases. Second, we need to define what we consider “normoganglionic” bowel since the simple presence of ganglion cells on H&E stain is not sufficient to ensure a normal density of ganglion cells and a normal ratio of excitatory and inhibitory neuronal subtypes. Acquiring normative data on healthy bowel at different ages and from different segments of the lower intestinal tract will be a key step toward refining our pathologic assessment of the transition zone. Finally, the surgical treatment of HSCR has not changed significantly over the past 70 years and remains based on removal of the aganglionic segment. While this is clearly problematic for infants with total intestinal aganglionosis, it is also suboptimal for all patients with HSCR, as it always requires removal of the rectum, which plays an important role in the maintenance of fecal continence. One scenario for the use of stem cell-based therapy is to repopulate the rectum with an enteric nervous system and thereby avoid a proctectomy at the time of surgery. Or, with further advances in cell therapy research, perhaps reinnervating the entire aganglionic segment, regardless of its length, will be feasible. New diagnostic tools are on the horizon and cell-based therapies may soon offer a novel treatment, but ongoing collaboration between fundamental scientists and pediatric surgeons is essential to bring these innovations to the bedside.

Disclosures

AMG receives research funding from Takeda Pharmaceuticals.

Reference

[1] Swenson O, Bill AH. Resection of rectum and rectosigmoid with preservation of the sphincter for benign spastic lesions producing megacolon; an experimental study. Surgery 1948;24(2):212–20.
[2] Amiel J, Lyonnet S. Hirschsprung disease, associated syndromes, and genetics: a review. J Med Genet 2001;38(11):729–39. doi:10.1136/jmg.38.11.729.
[3] Hukkinen M, Koivusalo A, Merras-Salmio L, et al. Postoperative outcome and survival in relation to small intestinal involvement of total colonic aganglionosis. J Pediatr Surg 2015;50(11):1859–64. doi:10.1016/j.jpedsurg.2015.05.017.
[4] Davidson JR, Kyrklund K, Eaton S, et al. Long-term surgical and patient-reported outcomes of Hirschsprung disease. J Pediatr Surg 2021;56(9):1502–11. doi:10.1016/j.jpedsurg.2021.01.043.
[5] Neuvonen MI, Kyrklund K, Rintala RJ, et al. Bowel function and quality of life after transanal endorectal pull-through for Hirschsprung disease: controlled outcomes up to adulthood. Ann Surg 2017;265(3):622–9. doi:10.1097/SLA.0000000000001695.
[6] Kapur RP, Raess PW, Hwang S, et al. Choline transporter immunohistochemistry: an effective substitute for acetylcholinesterase histochemistry to diagnose Hirschsprung disease with formalin-fixed paraffin-embedded rectal biopsies. Pediatr Dev Pathol 2017;20(4):308–20. doi:10.1177/1093526617697060.
[7] Holland SK, Ramalingam P, Podolsky RH, et al. Calretinin immunostaining as an adjunct in the diagnosis of Hirschsprung disease. Ann Diagn Pathol 2011;15(5):323–8. doi:10.1016/j.anndiagpath.2011.02.010.
[8] Gonzalo DH, Plesec T. Hirschsprung disease and use of calretinin in inadequate rectal suction biopsies. Arch Pathol Lab Med 2013;137(8):1099–102. doi:10.5858/arpa.2012-0220-OA.
[9] Kapur RP. Calretinin-immunoreactive mucosal innervation in very short-segment Hirschsprung disease: a potentially misleading observation. Pediatr Dev Pathol 2014;17(1):28–35. doi:10.2350/13-10-1387-oa.1.
[10] Galazka P, Szylberg L, Bodnar M, et al. Diagnostic algorithm in Hirschsprung's disease: focus on immunohistochemistry markers. In Vivo 2020;34(3):1355–9 (Brooklyn). doi:10.21873/invivo.11913.
[11] Heuckeroth RO. Hirschsprung disease-integrating basic science and clinical medicine to improve outcomes. Nat Rev Gastroenterol Hepatol 2018;15(3):152–67. doi:10.1038/nrgastro.2017.149.
[12] Teitelbaum DH, Cilley RE, Sherman NJ, et al. A decade of experience with the primary pull-through for hirschsprung disease in the newborn period: a multicenter analysis of outcomes. Ann Surg 2000;232(3):372–80. doi:10.1097/00000658-200009000-00009.
[13] Haikal Z, Dwihantoro A, Gunarti H, et al. Accuracy of transition zone in contrast enema to predict intraoperative aganglionosis level in patients with Hirschsprung disease. BMC Res Notes 2020;13(1):104. doi:10.1186/s13104-020-04945-2.
[14] Meinds RJ, Trzpis M, Broens PMA. Anorectal manometry may reduce the number of rectal suction biopsy procedures needed to diagnose Hirschsprung disease. J Pediatr Gastroenterol Nutr 2018;67(3):322–7. doi:10.1097/MPG.0000000000002000.
[15] Friedmacher F, Puri P. Rectal suction biopsy for the diagnosis of Hirschsprung's disease: a systematic review of diagnostic accuracy and complications. Pediatr Surg Int 2015;31(9):821–30. doi:10.1007/s00383-015-3742-8.
[16] Ambartsumyan L, Smith C, Kapur RP. Diagnosis of Hirschsprung disease. Pediatr Dev Pathol 2020;23(1):8–22. doi:10.1177/1093526619892351.
[17] Frykman PK, Lindsley EH, Gaon M, et al. Spectral imaging for precise surgical intervention in Hirschsprung's disease. J Biophotonics 2008;1(2):97–103. doi:10.1002/jbio.200710016.

[18] Dubois A, Vabre L, Boccara AC, et al. High-resolution full-field optical coherence tomography with a Linnik microscope. Appl Opt 2002;41(4):805–12. doi:10.1364/ao.41.000805.

[19] Auksorius E, Bromberg Y, Motiejūnaitė R, et al. Dual-modality fluorescence and full-field optical coherence microscopy for biomedical imaging applications. Biomed Opt Express 2012;3(3):661–6. doi:10.1364/boe.3.000661.

[20] Coron E, Auksorius E, Pieretti A, et al. Full-field optical coherence microscopy is a novel technique for imaging enteric ganglia in the gastrointestinal tract. Neurogastroenterol Motil 2012;24(12):e611–21. doi:10.1111/nmo.12035.

[21] Sumiyama K, Kiesslich R, Ohya TR, et al. *In vivo* imaging of enteric neuronal networks in humans using confocal laser endomicroscopy. Gastroenterology 2012;143(5):1152–3. doi:10.1053/j.gastro.2012.09.001.

[22] Kobayashi M, Sumiyama K, Shimojima N, et al. Technical feasibility of visualizing myenteric plexus using confocal laser endomicroscopy. J Gastroenterol Hepatol 2017;32(9):1604–10. doi:10.1111/jgh.13754.

[23] Shimojima N, Kobayashi M, Kamba S, et al. Visualization of the human enteric nervous system by confocal laser endomicroscopy in Hirschsprung's disease: an alternative to intraoperative histopathological diagnosis? Neurogastroenterol Motil 2020;32(5):e13805. doi:10.1111/nmo.13805.

[24] Granéli C, Erlöv T, Mitev RM, et al. Ultra high frequency ultrasonography to distinguish ganglionic from aganglionic bowel wall in Hirschsprung disease: a first report. J Pediatr Surg 2021. doi:10.1016/j.jpedsurg.2021.02.011.

[25] Cheng LS, Schwartz DM, Hotta R, et al. Bowel dysfunction following pullthrough surgery is associated with an overabundance of nitrergic neurons in Hirschsprung disease. J Pediatr Surg 2016;51(11):1834–8. doi:10.1016/j.jpedsurg.2016.08.001.

[26] Badner JA, Sieber WK, Garver KL, et al. A genetic study of Hirschsprung disease. Am J Hum Genet 1990;46(3):568–80 doi:.

[27] Tilghman JM, Ling AY, Turner TN, et al. Molecular genetic anatomy and risk profile of Hirschsprung's disease. N Engl J Med 2019;380(15):1421–32. doi:10.1056/NEJMoa1706594.

[28] Marsh SE, Blurton-Jones M. Neural stem cell therapy for neurodegenerative disorders: the role of neurotrophic support. Neurochem Int 2017;106:94–100. doi:10.1016/j.neuint.2017.02.006.

[29] McCann CJ, Cooper JE, Natarajan D, et al. Transplantation of enteric nervous system stem cells rescues nitric oxide synthase deficient mouse colon. Nat Commun 2017;8:15937. doi:10.1038/ncomms15937.

[30] Micci MA, Kahrig KM, Simmons RS, et al. Neural stem cell transplantation in the stomach rescues gastric function in neuronal nitric oxide synthase-deficient mice. Gastroenterology 2005;129(6):1817–24. doi:10.1053/j.gastro.2005.08.055.

[31] Fattahi F, Steinbeck JA, Kriks S, et al. Deriving human ENS lineages for cell therapy and drug discovery in Hirschsprung disease. Nature 2016;531(7592):105–9. doi:10.1038/nature16951.

[32] Rao M, Gershon MD. Enteric nervous system development: what could possibly go wrong? Nat Rev Neurosci 2018;19(9):552–65. doi:10.1038/s41583-018-0041-0.

[33] Natarajan DGM, Marcos-gutierrez CV, Atkins C, Pachnis V. Multipotential progenitors of the mammalian enteric nervous system capable of colonising aganglionic bowel in organ culture. Development 1999;126:10.

[34] Bondurand N, Natarajan D, Thapar N, et al. Neuron and glia generating progenitors of the mammalian enteric nervous system isolated from foetal and postnatal gut cultures. Development 2003;130(25):6387–400. doi:10.1242/dev.00857.

[35] Almond S, Lindley RM, Kenny SE, et al. Characterisation and transplantation of enteric nervous system progenitor cells. Gut 2007;56(4):489–96. doi:10.1136/gut.2006.094565.

[36] Kruger GM, Mosher JT, Bixby S, et al. Neural crest stem cells persist in the adult gut but undergo changes in self-renewal, neuronal subtype potential, and factor responsiveness. Neuron 2002;35(4):657–69. doi:10.1016/s0896-6273(02)00827-9.

[37] Cooper JE, McCann CJ, Natarajan D, et al. *In vivo* transplantation of enteric neural crest cells into mouse gut; engraftment, functional integration and long-term safety. PLoS One 2016;11(1):e0147989. doi:10.1371/journal.pone.0147989.

[38] Hotta R, Stamp LA, Foong JP, et al. Transplanted progenitors generate functional enteric neurons in the postnatal colon. J Clin Invest 2013;123(3):1182–91. doi:10.1172/JCI65963.

[39] Hetz S, Acikgoez A, Voss U, et al. *In vivo* transplantation of neurosphere-like bodies derived from the human postnatal and adult enteric nervous system: a pilot study. PLoS One 2014;9(4):e93605. doi:10.1371/journal.pone.0093605.

[40] Stamp LA, Gwynne RM, Foong JPP, et al. Optogenetic demonstration of functional innervation of mouse colon by neurons derived from transplanted neural cells. Gastroenterology 2017;152(6):1407–18. doi:10.1053/j.gastro.2017.01.005.

[41] Rollo BN, Zhang D, Stamp LA, et al. Enteric neural cells from Hirschsprung disease patients form ganglia in autologous aneuronal colon. Cell Mol Gastroenterol Hepatol 2016;2(1):92–109. doi:10.1016/j.jcmgh.2015.09.007.

[42] Cheng LS, Hotta R, Graham HK, et al. Postnatal human enteric neuronal progenitors can migrate, differentiate, and proliferate in embryonic and postnatal aganglionic gut environments. Pediatr Res 2017;81(5):838–46. doi:10.1038/pr.2017.4.

[43] Lindley RM, Hawcutt DB, Connell MG, et al. Human and mouse enteric nervous system neurosphere transplants regulate the function of aganglionic embryonic distal colon. Gastroenterology 2008;135(1):205–16 e206. doi:10.1053/j.gastro.2008.03.035.

[44] Hagl CI, Heumüller-Klug S, Wink E, et al. The human gastrointestinal tract, a potential autologous neural stem cell source. PLoS One 2013;8(9):e72948. doi:10.1371/journal.pone.0072948.

[45] Metzger M, Caldwell C, Barlow AJ, et al. Enteric nervous system stem cells derived from human gut mucosa for the treatment of aganglionic gut disorders. Gastroenterology 2009;136(7):2214–25 e2211-2213. doi:10.1053/j.gastro.2009.02.048.

[46] Wilkinson DJ, Bethell GS, Shukla R, et al. Isolation of enteric nervous system progenitor cells from the aganglionic gut of patients with Hirschsprung's disease. PLoS One 2015;10(5):e0125724. doi:10.1371/journal.pone.0125724.

[47] Cooper JE, Natarajan D, McCann CJ, et al. *In vivo* transplantation of fetal human gut-derived enteric neural crest cells. Neurogastroenterol Motil 2017;29(1). doi:10.1111/nmo.12900.

[48] Cheng LS, Graham HK, Pan WH, et al. Optimizing neurogenic potential of enteric neurospheres for treatment of neurointestinal diseases. J Surg Res 2016;206(2):451–9. doi:10.1016/j.jss.2016.08.035.

[49] Li XJ, Du ZW, Zarnowska ED, et al. Specification of motoneurons from human embryonic stem cells. Nat Biotechnol 2005;23(2):215–21. doi:10.1038/nbt1063.

[50] Lee G, Kim H, Elkabetz Y, et al. Isolation and directed differentiation of neural crest stem cells derived from human embryonic stem cells. Nat Biotechnol 2007;25(12):1468–75. doi:10.1038/nbt1365.

[51] Hotta R, Pepdjonovic L, Anderson RB, et al. Small-molecule induction of neural crest-like cells derived from human neural progenitors. Stem Cells 2009;27(12):2896–905. doi:10.1002/stem.208.

[52] Kawaguchi J, Nichols J, Gierl MS, et al. Isolation and propagation of enteric neural crest progenitor cells from mouse embryonic stem cells and embryos. Development 2010;137(5):693–704. doi:10.1242/dev.046896.

[53] Lai FP, Lau ST, Wong JK, et al. Correction of Hirschsprung-associated mutations in human induced pluripotent stem cells via clustered regularly interspaced short palindromic repeats/cas9, restores neural crest cell function. Gastroenterology 2017;153(1):139–53 e138. doi:10.1053/j.gastro.2017.03.014.

[54] Li W, Huang L, Zeng J, et al. Characterization and transplantation of enteric neural crest cells from human induced pluripotent stem cells. Mol Psychiatry 2018;23(3):499–508. doi:10.1038/mp.2016.191.

[55] Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 1992;255(5052):1707–10. doi:10.1126/science.1553558.

[56] Wagner JP, Sullins VF, Dunn JC. Transplanted skin-derived precursor stem cells generate enteric ganglion-like structures *in vivo*. J Pediatr Surg 2014;49(8):1319–24 discussion 1324-1315. doi:10.1016/j.jpedsurg.2014.01.061.

[57] Thomas AL, Taylor JS, Dunn JCY. Human skin-derived precursor cells xenografted in aganglionic bowel. J Pediatr Surg 2020. doi:10.1016/j.jpedsurg.2020.03.006.

[58] Kwok CK, Tam PK, Ngan ES. Potential use of skin-derived precursors (SKPs) in establishing a cell-based treatment model for Hirschsprung's disease. J Pediatr Surg 2013;48(3):619–28. doi:10.1016/j.jpedsurg.2012.08.026.

[59] Zhou Y, Besner G. Transplantation of amniotic fluid-derived neural stem cells as a potential novel therapy for Hirschsprung's disease. J Pediatr Surg 2016;51(1):87–91. doi:10.1016/j.jpedsurg.2015.10.016.

[60] Mortada I, Mortada R, Al Bazzal M. Dental pulp stem cells and neurogenesis. Adv Exp Med Biol 2018;1083:63–75. doi:10.1007/5584_2017_71.

[61] Gronthos S, Mankani M, Brahim J, et al. Postnatal human dental pulp stem cells (DPSCs) *in vitro* and *in vivo*. Proc Natl Acad Sci USA 2000;97(25):13625–30. doi:10.1073/pnas.240309797.

[62] Victor AK, Reiter LT. Dental pulp stem cells for the study of neurogenetic disorders. Hum Mol Genet 2017;26(R2):R166–71. doi:10.1093/hmg/ddx208.

[63] Nuti N, Corallo C, Chan BM, et al. Multipotent differentiation of human dental pulp stem cells: a literature review. Stem Cell Rev Rep 2016;12(5):511–23. doi:10.1007/s12015-016-9661-9.

[64] Arthur A, Rychkov G, Shi S, et al. Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues. Stem Cells 2008;26(7):1787–95. doi:10.1634/stemcells.2007-0979.

[65] Sakai K, Yamamoto A, Matsubara K, et al. Human dental pulp-derived stem cells promote locomotor recovery after complete transection of the rat spinal cord by multiple neuro-regenerative mechanisms. J Clin Invest 2012;122(1):80–90. doi:10.1172/JCI59251.

[66] Bettolli M, De Carli C, Jolin-Dahel K, et al. Colonic dysmotility in postsurgical patients with Hirschsprung's disease. Potential significance of abnormalities in the interstitial cells of Cajal and the enteric nervous system. J Pediatr Surg 2008;43(8):1433–8. doi:10.1016/j.jpedsurg.2007.10.067.

[67] Won KJ, Torihashi S, Mitsui-Saito M, et al. Increased smooth muscle contractility of intestine in the genetic null of the endothelin ETB receptor: a rat model for long segment Hirschsprung's disease. Gut 2002;50(3):355–60. doi:10.1136/gut.50.3.355.

[68] Thiagarajah JR, Yildiz H, Carlson T, et al. Altered goblet cell differentiation and surface mucus properties in Hirschsprung disease. PLoS One 2014;9(6):e99944. doi:10.1371/journal.pone.0099944.

[69] Medrano G, Cailleux F, Guan P, et al. B-lymphocyte-intrinsic and -extrinsic defects in secretory immunoglobulin A production in the neural crest-conditional deletion of endothelin receptor B model of Hirschsprung-associated enterocolitis. FASEB J 2019;33(6):7615–24. doi:10.1096/fj.201801913R.

[70] Neuvonen MI, Korpela K, Kyrklund K, et al. Intestinal microbiota in Hirschsprung disease. J Pediatr Gastroenterol Nutr 2018;67(5):594–600. doi:10.1097/MPG.0000000000001999.

[71] Bhave S, Arciero E, Baker C, et al. Enteric neuronal cell therapy reverses architectural changes in a novel diphtheria toxin-mediated model of colonic aganglionosis. Sci Rep 2019;9(1):18756. doi:10.1038/s41598-019-55128-4.
[72] Mosher JT, Yeager KJ, Kruger GM, et al. Intrinsic differences among spatially distinct neural crest stem cells in terms of migratory properties, fate determination, and ability to colonize the enteric nervous system. Dev Biol 2007;303(1):1–15. doi:10.1016/j.ydbio.2006.10.026.
[73] Hotta R, Cheng LS, Graham HK, et al. Isogenic enteric neural progenitor cells can replace missing neurons and glia in mice with Hirschsprung disease. Neurogastroenterol Motil 2016;28(4):498–512. doi:10.1111/nmo.12744.
[74] Mwizerwa O, Das P, Nagy N, et al. Gdnf is mitogenic, neurotrophic, and chemoattractive to enteric neural crest cells in the embryonic colon. Dev Dyn 2011;240(6):1402–11. doi:10.1002/dvdy.22630.
[75] Nagy N, Barad C, Hotta R, et al. Collagen 18 and agrin are secreted by neural crest cells to remodel their microenvironment and regulate their migration during enteric nervous system development. Development 2018;145(9). doi:10.1242/dev.160317.
[76] Hotta R, Anderson RB, Kobayashi K, et al. Effects of tissue age, presence of neurones and endothelin-3 on the ability of enteric neurone precursors to colonize recipient gut: implications for cell-based therapies. Neurogastroenterol Motil 2010;22(3):331–86. doi:10.1111/j.1365-2982.2009.01411.x.
[77] Binder E, Natarajan D, Cooper J, et al. Enteric neurospheres are not specific to neural crest cultures: implications for neural stem cell therapies. PLoS One 2015;10(3):e0119467. doi:10.1371/journal.pone.0119467.
[78] Zhang D, Rollo BN, Nagy N, et al. The enteric neural crest progressively loses capacity to form enteric nervous system. Dev Biol 2019;446(1):34–42. doi:10.1016/j.ydbio.2018.11.017.
[79] McKeown SJ, Mohsenipour M, Bergner AJ, et al. Exposure to GDNF enhances the ability of enteric neural progenitors to generate an enteric nervous system. Stem Cell Rep 2017;8(2):476–88. doi:10.1016/j.stemcr.2016.12.013.
[80] Hegewald C, Alt R, Hetz S, et al. Reduced oxygen stress promotes propagation of murine postnatal enteric neural progenitors *in vitro*. Neurogastroenterol Motil 2011;23(10):e412–24. doi:10.1111/j.1365-2982.2011.01761.x.
[81] Hotta R, Cheng L, Graham HK, et al. Delivery of enteric neural progenitors with 5-HT4 agonist-loaded nanoparticles and thermosensitive hydrogel enhances cell proliferation and differentiation following transplantation *in vivo*. Biomaterials 2016;88:1–11. doi:10.1016/j.biomaterials.2016.02.016.
[82] Sato Y, Heuckeroth RO. Retinoic acid regulates murine enteric nervous system precursor proliferation, enhances neuronal precursor differentiation, and reduces neurite growth *in vitro*. Dev Biol 2008;320(1):185–98. doi:10.1016/j.ydbio.2008.05.524.
[83] Barlow A, de Graaff E, Pachnis V. Enteric nervous system progenitors are coordinately controlled by the G protein-coupled receptor EDNRB and the receptor tyrosine kinase RET. Neuron 2003;40(5):905–16. doi:10.1016/s0896-6273(03)00730-x.
[84] Schuster A, Klotz M, Schwab T, et al. Granulocyte-colony stimulating factor: a new player for the enteric nervous system. Cell Tissue Res 2014;355(1):35–48. doi:10.1007/s00441-013-1744-1.
[85] Gisser JM, Cohen AR, Yin H, et al. A novel bidirectional interaction between endothelin-3 and retinoic acid in rat enteric nervous system precursors. PLoS One 2013;8(9):e74311. doi:10.1371/journal.pone.0074311.
[86] Stavely R, Bhave S, Ho WLN, et al. Enteric mesenchymal cells support the growth of postnatal enteric neural stem cells. Stem Cells 2021. doi:10.1002/stem.3388.
[87] Barber K, Studer L, Fattahi F. Derivation of enteric neuron lineages from human pluripotent stem cells. Nat Protoc 2019;14(4):1261–79. doi:10.1038/s41596-019-0141-y.
[88] 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(1):49–59. doi:10.1038/nm.4233.
[89] Semrau S, Goldmann JE, Soumillon M, et al. Dynamics of lineage commitment revealed by single-cell transcriptomics of differentiating embryonic stem cells. Nat Commun 2017;8(1):1096. doi:10.1038/s41467-017-01076-4.
[90] Lau ST, Li Z, Pui-Ling Lai F, et al. Activation of hedgehog signaling promotes development of mouse and human enteric neural crest cells, based on single-cell transcriptome analyses. Gastroenterology 2019;157(6):1556–71 e1555. doi:10.1053/j.gastro.2019.08.019.
[91] Evans GS, Flint N, Somers AS, et al. The development of a method for the preparation of rat intestinal epithelial cell primary cultures. J Cell Sci 1992;101(Pt 1):219–31 doi:.
[92] Grikscheit TC, Siddique A, Ochoa ER, et al. Tissue-engineered small intestine improves recovery after massive small bowel resection. Ann Surg 2004;240(5):748–54. doi:10.1097/01.sla.0000143246.07277.73.
[93] Sala FG, Matthews JA, Speer AL, et al. A multicellular approach forms a significant amount of tissue-engineered small intestine in the mouse. Tissue Eng Part A 2011;17(13–14):1841–50. doi:10.1089/ten.TEA.2010.0564.
[94] Sala FG, Kunisaki SM, Ochoa ER, et al. Tissue-engineered small intestine and stomach form from autologous tissue in a preclinical large animal model. J Surg Res 2009;156(2):205–12. doi:10.1016/j.jss.2009.03.062.
[95] Levin DE, Barthel ER, Speer AL, et al. Human tissue-engineered small intestine forms from postnatal progenitor cells. J Pediatr Surg 2013;48(1):129–37. doi:10.1016/j.jpedsurg.2012.10.029.
[96] DiMarco RL, Su J, Yan KS, et al. Engineering of three-dimensional microenvironments to promote contractile behavior in primary intestinal organoids. Integr Biol 2014;6(2):127–42 (Camb). doi:10.1039/c3ib40188j.
[97] Chen Y, Zhou W, Roh T, et al. *In vitro* enteroid-derived three-dimensional tissue model of human small intestinal epithelium with innate immune responses. PLoS One 2017;12(11):e0187880. doi:10.1371/journal.pone.0187880.
[98] Ueda T, Yamada T, Hokuto D, et al. Generation of functional gut-like organ from mouse induced pluripotent stem cells. Biochem Biophys Res Commun 2010;391(1):38–42. doi:10.1016/j.bbrc.2009.10.157.
[99] Takaki M, Nakayama S, Misawa H, et al. *In vitro* formation of enteric neural network structure in a gut-like organ differentiated from mouse embryonic stem cells. Stem Cells 2006;24(6):1414–22. doi:10.1634/stemcells.2005-0394.
[100] Spence JR, Mayhew CN, Rankin SA, et al. Directed differentiation of human pluripotent stem cells into intestinal tissue *in vitro*. Nature 2011;470(7332):105–9. doi:10.1038/nature09691.
[101] Schlieve CR, Fowler KL, Thornton M, et al. Neural crest cell implantation restores enteric nervous system function and alters the gastrointestinal transcriptome in human tissue-engineered small intestine. Stem Cell Rep 2017;9(3):883–96. doi:10.1016/j.stemcr.2017.07.017.