Review

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Recent developments in cell-based ENS regeneration – a short review

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Abstract: Therapeutic options to treat neurogenic motility disorders of the gastrointestinal tract are usually limited to symptomatic treatment. The capacity of the enteric nervous system (ENS) to regenerate and the fact that progenitor cells of the enteric nervous system reside in the postnatal and adult gut led to the idea to develop cell-based strategies to treat ENS related disorders. This short review focuses on recent developments in cell-based ENS regeneration, discussing advantages and disadvantages of various cell sources, functional impact of transplanted cells and highlights the challenges of translation of small animal studies to human application.

Keywords: cell therapy; enteric nervous system; Hirschsprung disease; regeneration; stem cells.

Introduction

The enteric nervous system (ENS) regulates various functions of the gastrointestinal tract, such as motility, blood flow, secretion and fluid exchange, and modulates the immune system of the gut [1–3]. The ENS derives mainly from vagal neural crest cells that enter the foregut during development and migrate along the gut, in order to colonize the whole GI tract, forming an interconnected network of neurons and glial cells [4–6]. During neural colonization of the growing GI tract, processes such as proliferation, migration and differentiation of enteric neural crest cells take place in parallel, orchestrated by a complex program of genes [7, 8]. Dysregulation of these processes leads to either qualitative changes in ENS composition or to quantitative alteration of the number of ENS cells within the gut. One of the most prominent developmental disorders of the ENS is Hirschsprung disease (HSCR). HSCR is defined by a complete loss of neural crest-derived neurons and glial cells in the distal part of the colon, which results in chronic constipation, ileus, enterocolitis and failure to thrive [9]. In addition to congenital disorders of the ENS, many acquired and degenerative changes of the ENS can impair bowel function in children and adults [10]. Therapeutic options are limited for both developmental and acquired ENS disorders. In HSCR, surgical resection of the affected gut segment and colo-anal anastomosis lead to cure in many patients with short-segment disease, but are is associated with numerous long-term complications in those with syndromic or long-segment disease [10, 11].

Since therapeutic options are limited and quality of life appears to be impaired in a relevant proportion of patients with neurogenic motility disorders of the gut, a regenerative therapeutic approach was proposed many years ago [12–14]. The fact that stem or progenitor cells of the ENS also reside in the postnatal and adult gut in both animal models and humans led to the idea to isolate stem or progenitor cells of the ENS, to expand them *in vitro* and to re-implant them into the affected gut, in order to rehabilitate gut function.

During the last years, much effort was put into defining the ENS stem cell niche in animals and humans, and isolation and expansion protocols for ENS stem or progenitor cells were developed and transplantation of these cells in *in vitro* and *in vivo* models was performed, mainly demonstrating survival and anatomic and partially functional integration of transplanted cells into the host gut [10]. However, many aspects of a cell-based approach remain to be elucidated yet [15]. This short review summarizes recent progress in the field of ENS regeneration focusing on the cell source, *in vitro* expansion, functional impact of ENS transplantation and technical aspects of cell delivery. Since HSCR is well defined from a genetic and clinical point of view, and numerous small animal models for HSCR exist, most of the research was performed focusing on regeneration the ENS of HSCR animal models in the past.

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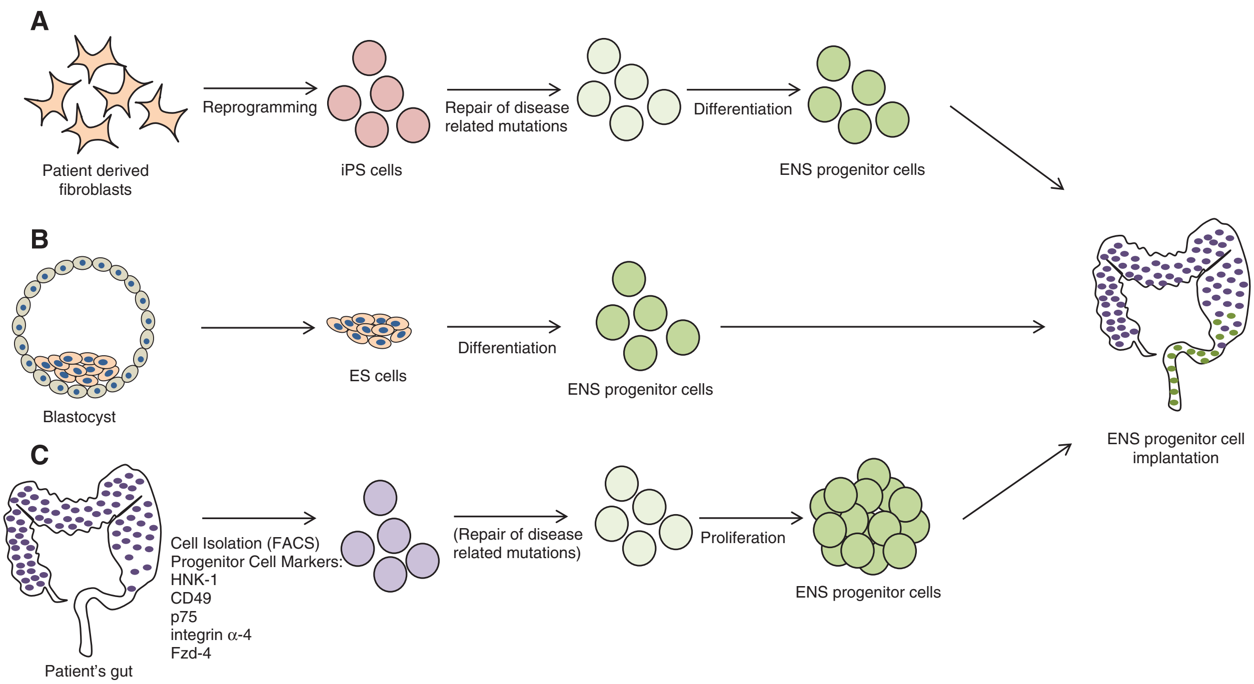

Figure 1: Schematic view of the important steps of ENS progenitor generation and transplantation.
(A) Generation of ENS progenitor cells from patient-derived induced pluripotent stem (iPS) cells. Somatic cell can be reprogrammed to generate iPS cells, which can be differentiated into ENS progenitor cells [16]. Since the cells are patient-derived, disease-associated gene mutation might limit their proliferative, migratory and differentiation behavior. Thus, repair of the mutations might be necessary to rescue these defects, as described [16]. (B) Generation of ENS progenitor cells from pluripotent embryonic stem cells (ES cells) has been described by Fattahi et al. [17]. They were even able to demonstrate rescue of a HSCR mouse model by transplantation of generated ENS progenitors into the cecum of neonatal mice. (C) Generation of ENS progenitor cells derived from the patient's gut. Isolation of the proper cell types relies on progenitor cell-specific cell surface markers. In contrast to ES or iPS cells, the self-renewal capacity of these cells is limited. Thus, optimizing the *in vitro* expansion condition is an important step in generating a sufficient number of cells that allows colonization of a large area.

Cell source

The optimal cell source for ENS cell transplantation can be defined as easily accessible; harvested cells should proliferate *in vitro* to an extent that is sufficient to colonize the defective gut segment. The cells should migrate into the correct position after transplantation and differentiate into proper cell types in the recipient gut, generating an interconnected network of neurons and glial cells. No adverse effects, such as tumor formation or graft rejection, should be associated with cell transplantation.

Cells of various anatomic origin and developmental stages have been proposed to serve as cell source for ENS cell transplantation. While ENS progenitor cells were generated from numerous tissues in the past, not all fulfill the above-mentioned criteria, and some, like central nervous system (CNS) cells, are so difficult to access that they are *a priori* not suitable for human application [12–14].

There are mainly three sources of cells to generate ENS progenitors that represent the currently most promising candidates: patient-derived ENS progenitors isolated from the gut, embryonic pluripotent stem cells (ES), and induced pluripotent stem cells (iPS). The advantages and disadvantages of these cell sources are discussed below (Figure 1).

Patient-derived ENS progenitor cells from the gut

It is generally accepted that progenitor cells of the ENS persist also in the postnatal gut of animals and humans [10]. Many studies demonstrated successful isolation and *in vitro* propagation of ENS progenitors from rodents and humans of various ages [18–22]. The use of these patient-derived cells is associated with the advantage that autologous cell transplantation can be performed and no immunosuppression needs to be initiated after transplantation in order to prevent host-versus-graft disease. In addition, the cells are easily accessible either by laparoscopic procedures [23] or by endoscopic suction biopsies [24], although the amount of tissue that can be taken is limited. Progenitor cells isolated from postnatal gut were shown to differentiate into functional active enteric neurons and into glial cells when transplanted into the gut of rodents [19, 20, 25]. However, postnatally generated progenitor cells from the gut have a reduced capacity for self-renewal, and even though they can be passaged several times, they have been shown to lose their progenitor cell state over time, which represents a major problem concerning cell expansion *in vitro* [26]. Another fact that may also contribute to reduced proliferative potential are disease-related gene mutations.

To overcome the problem of the low proliferation rate and reduced ENS progenitor cell expansion *in vitro*, several strategies to optimize ENS progenitor cell generation from the postnatal gut have been evaluated.

The basic requirement for an optimal yield of ENS progenitor cells is to isolate the most appropriate cell types from the gut. Although in small animal studies, genetically labeled ENS progenitor cells can be used, this approach is not suitable for human application. Cell isolation from human tissue either relies on sorting of cells for distinct cell surface markers or on selective culture conditions that permit proliferation of mainly neural progenitor cells. Although such permissive culture conditions lead to an enrichment of neural progenitors, which often form so called neurosphere like bodies, as their counterparts from the CNS, many other cell types such as fibroblasts or smooth muscle cells can be found in these cultures [19, 27]. Attempting to enrich primarily isolated cells for ENS progenitors in humans or mice, numerous cell surface antigens have been proposed to serve as markers for selective neural progenitor cells isolation from the gut such as HNK-1 [28], p75 [29, 30], integrin α-4 [31] and CD49 [32]. Although a concentration of proliferating neural cells *in vitro* can be achieved by cell sorting, the combination of markers that will isolate the highest and purest amount of enteric neural progenitors is still unknown. We recently found Fizzled-4, a Wnt receptor, to define a subpopulation of p75-sorted cells [33]. Preliminary data demonstrate that only p75+/Fzd4+, but not p75+/Fzd4– cells proliferate *in vitro*. Thus, Fzd4 defines a subpopulation of human p75+ cells that might represent a purer population of ENS progenitor cells. Whether such attempts to enrich ENS progenitors will eventually lead to a significant improvement of *in vitro* cell expansion needs to be further investigated.

Optimizing cell culture conditions is another way to increase cell numbers prior to transplantation. ENS progenitors are often grown in culture medium supplemented with growth factors like fibroblast growth factor and epidermal growth factor. In addition, other factors such as glial cell-derived neurotrophic factor [34, 35], granulocyte colony-stimulating factor [36], bacterial lipopolysaccharides [37] or endothelin 3 [35] have been shown to positively influence proliferation or stemness of cultured cells. In recent studies the importance of the Wnt signaling pathway in proliferating ENS progenitors has been described. Neckel et al. [38] performed microarray analysis and found Wnt signaling to be turned off when NBLs stop to proliferate and start to differentiate. In another study, a positive impact of Wnt agonists on neurosphere growth was demonstrated [39]. This complies with the observation of Rollo et al. [30], who found ENS progenitors isolated from HSCR patients to be restricted in their proliferative potential, which could be partially rescued by chemical Wnt stimulation.

Although numerous studies have improved cell-isolation techniques and cell culture conditions, and novel genetic techniques are available to manipulate the isolated cells, it remains unclear if the postnatal gut will be the optimal cell source for ENS regeneration, in particular concerning generation of a sufficient cell number.

Embryonic pluripotent stem cells

The most striking characteristics of ES cells are their capacity for near unlimited self-renewal and that they can give rise to nearly any cell type, given that an appropriate differentiation protocol is established [17]. Thus, ES cells are more likely to generate a relevant cell number, compared with postnatal gut-derived cells, as discussed above. However, there are also some disadvantages that are associated with the use of ES cells that need to be addressed before application in human disease. Importantly, the use of embryonic tissue for human therapeutic applications remains ethically problematic and it is unknown if ES cells will be available for this purpose in the future [40]. In addition, ES cells are usually not patient-derived; thus, host-versus-graft reaction will occur or immunosuppressant therapy will be necessary. Moreover, tumor formation has been an issue in ES cell transplantation. Although differentiated ES-derived cells are not supposed to result in tumor formation, accidental co-transplantation of not fully or undifferentiated ES cells are suspected to produce teratoma [41]. Therefore, reliable cell selection strategies need to be established to prevent transplantation of immature cells. Besides host-versus-graft reactions and ethical issues, the safety of ES cell-derived ENS progenitor cells need to be further examined.

Induced pluripotent stem cells

Reprogramming of adult somatic cells allows turning them into iPS with also near-limitless self-renewal capacity and pluripotency. However, as in ES cells, the safety aspects need to be taken into consideration before application in human therapy to avoid tumor formation within the recipient. In contrast to ES cells, iPS can be generated from patient-derived tissue; thus, autologous transplantation is possible, making immunosuppressive therapy unnecessary. However, as for gut-derived progenitor cells, autologous iPS might also be affected by disease-related gene mutation as demonstrated recently. Lai et al. [16] generated iPS from patients with HSCR and differentiated them into ENS progenitor cells. Compared with iPS-derived ENS progenitors generated from non-HSCR patients, HSCR patient-derived progenitor cells showed significantly impaired differentiation and migration characteristics, which interestingly could be reversed by repairing underlying gene mutations.

Integration of ENS progenitor cells into recipient gut

ENS cell transplantation experiments were performed mainly in wild-type mice or rats with an intact ENS [27, 42, 43]. These studies consistently demonstrated survival and migration of transplanted cells as well as their differentiation into various neuron subtypes and glial cells and formation of interconnected ganglion-like structures within the myenteric plexus using both embryonic and postnatal murine ENS progenitor cells. Moreover, specific electric activity could be observed in neurons derived from transplanted ENS progenitors, which respond to electrical stimulation, fire action potentials and receive input from other neurons *via* synaptic connections [42]. In addition, introduction of optogenetic techniques allowed Stamp et al. [44] to further dissect functional integration of transplanted cells. They were able to demonstrate that light-dependent stimulation of transplanted cells lead to excitatory and inhibitory neuronal responses and were also able to identify graft derived interneurons within the recipient gut *in vivo*. As mentioned above, these experiments were performed in animals with an intact ENS, which might serve as a scaffold for transplanted cells. However, in the aganglionic gut of HSCR patients for example, such a scaffold will not be present. Thus, it is unclear, whether the microenvironment of the aganglionic gut will support functional integration of transplanted ENS progenitors. Preliminary studies have shown survival, migration and differentiation of murine ENS progenitor cells when transplanted into *in vivo* HSCR mouse models. Whether this is as effective as in wild-type animals remains to be demonstrated [45].

Transplantation of human ENS progenitor cells was first performed *in vitro* organ/tissue culture, since immunological issues do not allow transplantation into immune competent mice. Lindley et al. [20] implanted neurosphere-like bodies (NLBs) generated from neonatal human colon into aganglionic embryonic mouse gut. As Metzger et al. [19], they demonstrated neuronal differentiation of implanted cells. *In vivo* transplantation experiments were performed with immunocompromised mouse models with and without wild-type ENS. Hetz et al. [25] implanted ENS progenitor cells that were generated from the postnatal human gut into immunocompromised mice, in which parts of the ENS were destroyed chemically before implantation. Although neuronal and glial differentiation could be demonstrated, the functional impact on gut motility remained unclear. Cheng et al. [46] even performed implantation of human ENS progenitor cells into the aganglionic segment of a HSCR mouse model. They were able to demonstrate survival, migration and differentiation of implanted cells into neurons, but the functional impact of implanted cells could not yet be demonstrated.

In intriguing recent studies, human ENS progenitor cells of embryonic origin were implanted into the mouse colon of genetically generated models of ENS motility disorders. Fattahi et al. [17] transplanted ES cell-derived ENS progenitors into the cecum of a HSCR mouse model and were able to show complete colonization of the recipient colon with the transplanted cells. Mouse models for HSCR usually die within 3–4 weeks after birth. Excitingly, the authors demonstrated that cell transplantation was able to prevent mortality and restore colonic motility. Although this is the first study in which HSCR mice could be rescued by cell transplantation, cellular and subcellular mechanisms achieving these effects were not demonstrated.

McCann et al. [47] isolated ENS progenitor cells from the fetal human gut and transplanted them into wild-type and nitric oxide synthase (NOS)-deficient mice. They found transplanted cells in about 50% of the recipient animals, which was attributed to the variability of donor tissue. Graft-derived cells differentiated into functional active neurons that responded to electrical stimulation, and even more excitingly, they were able to restore NOS-dependent function in NOS-deficient mice.

Cell delivery techniques

Reasonable progress has been made concerning generation of ENS progenitor cells and demonstrating their potential to restore gastrointestinal motility in small animals, but successful cell transplantation in humans will depend not only on the biological and genetic characteristics of the donor and the recipient, but also on practical aspects, such as suitable cell delivery techniques. In mice, cells are usually injected into the gut wall as suspension [43] or single NLB are introduced into subserosal pockets [42]. Although these implantation techniques have been shown to result in relevant colonization of the adjacent tissue, it is not supposed that these techniques of single-site implantation will result in the colonization of a relevant gut segment in humans, which will be disproportionately larger than in small animals. New techniques need to be developed to efficiently transplant the cells in a rather atraumatic fashion. Cheng et al. [48], for example, demonstrated injection of ENS progenitors *via* the endoscopic route into the colon of a HSCR mouse model. They were able to find the cells in 9/12 injected mice, but implanted cells were mainly located along the subserosal plane and did not migrate into the myenteric plexus region. This is in keeping with previous studies in which ENS progenitor cells were implanted into the myenteric plexus. In these experiments, only extensive longitudinal and circumferential migration of transplanted cells within the gut wall was observed, but no or only rudimentary centripetal or centrifugal migration of the transplanted cells [42, 43]. Thus, layer-specific delivery of cells appears to be important and needs to be developed to translate the murine experiments into human application. In addition, only few studies were performed examining the effect of co-transplantation of other cell types or growth, differentiation or chemotactic factors on graft survival, *in vivo* proliferation, differentiation and network formation *in vivo*. First studies, for example, indicate that adding serotonin agonists during transplantation results in a higher density of neurons *in vitro* and *in vivo* in the mouse or rat gut [49, 50]. Thus, new techniques need to be developed to enable large-area transplantation of ENS progenitors in the future.

Conclusion

Therapeutic options are limited for neurogenic disorders of the gut. Cell-based treatment strategies are promising, taking recent developments into account. However, many aspects need to be addressed before human application is possible, such as detailed examination of the mechanisms leading to functional regeneration, further investigation of safety aspects and surgical delivery techniques that allow colonization of large gut segments have to be developed. The research on ENS stem or progenitor cell therapy mainly focuses on motility; however, restoration of motility is only one aspect, and the other functions of the ENS should not be neglected when investigating the effects of ENS transplantation.

Author Statement
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Author Contributions
Florian Obermayr: conceptualization; investigation; writing – original draft; writing – review and editing. Guido Seitz: writing – original draft; writing – review and editing.

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Reviewer Assessment

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Reviewers' Comments to Original Submission

Reviewer 1: anonymous

Feb 11, 2018

Reviewer Recommendation Term:	Accept
Overall Reviewer Manuscript Rating:	85
Custom Review Questions	**Response**
Is the subject area appropriate for you?	5 - High/Yes
Does the title clearly reflect the paper's content?	5 - High/Yes
Does the abstract clearly reflect the paper's content?	5 - High/Yes
Do the keywords clearly reflect the paper's content?	5 - High/Yes
Does the introduction present the problem clearly?	5 - High/Yes
Are the results/conclusions justified?	5 - High/Yes
How comprehensive and up-to-date is the subject matter presented?	4
How adequate is the data presentation?	4
Are units and terminology used correctly?	5 - High/Yes
Is the number of cases adequate?	N/A
Are the experimental methods/clinical studies adequate?	N/A
Is the length appropriate in relation to the content?	4
Does the reader get new insights from the article?	4
Please rate the practical significance.	1 - Low/No
Please rate the accuracy of methods.	N/A
Please rate the statistical evaluation and quality control.	N/A
Please rate the appropriateness of the figures and tables.	4
Please rate the appropriateness of the references.	4
Please evaluate the writing style and use of language.	4
Please judge the overall scientific quality of the manuscript.	4
Are you willing to review the revision of this manuscript?	Yes

Comments to Authors:
This is an invited review on recent developments in cell-based ENS regeneration. Under these circumstances, the review is perfectly appropriate. The authors describe the current methods and results in lab-based ENS regeneration. Naturally, these approaches lack a clear clinical significance. As an invited review, this manuscript perfectly meets the requirements.

Reviewer 2: anonymous

Feb 07, 2018

Reviewer Recommendation Term:	Accept with Minor Revision
Overall Reviewer Manuscript Rating:	85
Custom Review Questions	**Response**
Is the subject area appropriate for you?	5 - High/Yes
Does the title clearly reflect the paper's content?	4
Does the abstract clearly reflect the paper's content?	4
Do the keywords clearly reflect the paper's content?	5 - High/Yes
Does the introduction present the problem clearly?	3
Are the results/conclusions justified?	4
How comprehensive and up-to-date is the subject matter presented?	5 - High/Yes
How adequate is the data presentation?	N/A
Are units and terminology used correctly?	5 - High/Yes
Is the number of cases adequate?	N/A
Are the experimental methods/clinical studies adequate?	N/A
Is the length appropriate in relation to the content?	4
Does the reader get new insights from the article?	4
Please rate the practical significance.	3
Please rate the accuracy of methods.	N/A
Please rate the statistical evaluation and quality control.	N/A
Please rate the appropriateness of the figures and tables.	N/A
Please rate the appropriateness of the references.	4
Please evaluate the writing style and use of language.	4
Please judge the overall scientific quality of the manuscript.	3
Are you willing to review the revision of this manuscript?	Yes

Comments to Authors:
Recent developments in cell-based ENS regeneration - a short review.
The author described all cell sources to generate ENS progenitor cells and their application in different animal or human in vitro models. A separate paragraph is focused on delivery techniques of the ENS progenitor cells into the colon.
He discussed specific details of each model and their implication for ENS regeneration in humans.

Some comments:
1. Within the introduction the author mentioned that the field of application for ENS regeneration comprises developmental diseases such as Hirschsprung disease (HSCR) on the one hand and acquired degenerative disorders on the other. But the following exposition are considered on the potentially HSCR treatment by ENS regeneration only. Are there any studies investigating ENS regeneration in degenerative disorders?
2. More the 80% of the Hirschsprung disease patient are suffering from short segment aganglionosis causing a recto-sigmoid resection - no complete colectomy - which results in a really good outcome in most cases. Therefore, I cannot share the disastrous picture of the HSCR treatment in the manuscript.
3. The paragraph about iPS cells should include a few sentences that discuss safety problems of iPS cells and how to circumvent them.
4. There are several typing errors.

Authors' Response to Reviewer Comments

Feb 15, 2018

Dear Professor Jaehne,
thank you for sending the reviewers' comments. We've revised the manuscript accordingly:

Reviewer I: no changes.

Reviewer II:

1. Are there any studies investigating ENS regeneration in degenerative disorders?

There are some studies investigating cell-based regeneration in animal models different to a Morbus Hirschsprung pheno- or genotype. However Hirschsprungs disease represents the model disease for this research, thus most of the research on this topic was done on Hirschprung animals. Therefore we introduced a sentence that clarifies this in the introduction section:

“Since HSCR is well defined from a genetic and clinical point of view, and numerous small animal models for HSCR exist, most of the research was performed focusing on regeneration the ENS of HSCR animal models in the past. “

2. Most of the patients treated for HSCR suffer from short-segment disease and have a good surgical outcome!

We agree with the reviewer, that surgical outcome in a proportion of patients is good after surgical intervention. However literature is contradictionary, mainly concerning functional outcome. Detailed review of surgical outcome of surgery for Hirschprung disease is beyond the scope of the manuscript. Accordingly we changed the paragraph dealing with HSCR outcome:

“Therapeutic options are limited for both, developmental and acquired ENS disorders. In HSCR surgical resection of the affected gut segment and colo-anal anastomosis leads to cure in many patients with short-segment disease, but is associated with numerous long-term complications in those suffering from syndromic or long-segment disease (10,11).

Since therapeutic options are limited and quality of life appears to be impaired in a relevant proportion of patients suffering from neurogenic motility disorders of the gut, a regenerative therapeutic approach was proposed many years ago (12-14).”

3. iPS cell safety issues:

We agree that safety issues are important, teratoma formation in particular. This was mentioned already in the ES cell section, to which we referenced in the iPS cell section already. To underline the importance of this problem we added:

“Therefore, reliable cell selection strategies need to be established in order to prevent transplantation of immature cells.”

and

“…avoiding tumor formation within the recipient.”

4. The manuscript was checked for typing errors.

We hope the manuscript will be appropriate now for publication.

With best regards

Reviewers’ Comments to Revision

Reviewer 2: anonymous

Feb 15, 2018

Reviewer Recommendation Term:	Accept
Overall Reviewer Manuscript Rating:	N/A
Custom Review Questions	**Response**
Is the subject area appropriate for you?	5 - High/Yes
Does the title clearly reflect the paper’s content?	4
Does the abstract clearly reflect the paper’s content?	4
Do the keywords clearly reflect the paper’s content?	5 - High/Yes
Does the introduction present the problem clearly?	4
Are the results/conclusions justified?	4
How comprehensive and up-to-date is the subject matter presented?	5 - High/Yes
How adequate is the data presentation?	N/A
Are units and terminology used correctly?	5 - High/Yes
Is the number of cases adequate?	N/A
Are the experimental methods/clinical studies adequate?	N/A
Is the length appropriate in relation to the content?	4

Does the reader get new insights from the article?	4
Please rate the practical significance.	3
Please rate the accuracy of methods.	N/A
Please rate the statistical evaluation and quality control.	N/A
Please rate the appropriateness of the figures and tables.	N/A
Please rate the appropriateness of the references.	4
Please evaluate the writing style and use of language.	4
Please judge the overall scientific quality of the manuscript.	3
Are you willing to review the revision of this manuscript?	Yes

Comments to Authors:
I have no further comments. I suggest accepting the manuscript for publication ISS.