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White paper on guidelines concerning enteric nervous system stem cell therapy for enteric neuropathies ☆

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ABSTRACT

Over the last 20 years, there has been increasing focus on the development of novel stem cell based therapies for the treatment of disorders and diseases affecting the enteric nervous system (ENS) of the gastrointestinal tract (so-called enteric neuropathies). Here, the idea is that ENS progenitor/stem cells could be transplanted into the gut wall to replace the damaged or absent neurons and glia of the ENS. This White Paper sets out experts' views on the commonly used methods and approaches to identify, isolate, purify, expand and optimize ENS stem cells, transplant them into the bowel, and assess transplant success, including restoration of gut function. We also highlight obstacles that must be overcome in order

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to progress from successful preclinical studies in animal models to ENS stem cell therapies in the clinic.

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0. Introduction

This white paper, authored by 30 members of the enteric nervous system (ENS) basic science and clinical field, sets out their opinions on efforts to establish novel stem cell therapies for enteric neuropathies of the gastrointestinal tract. Such enteric neuropathies remain some of the most challenging clinical disorders to manage. Arguably the best understood enteric neuropathy is the congenital disorder Hirschsprung disease (HSCR) in which the neural crest-derived intrinsic ENS is absent in a variable length of the distal gut (called "aganglionosis"). The only treatment currently available for HSCR is surgical removal of the aganglionic bowel segment, and although life saving, chronic gastrointestinal problems, including faecal incontinence and enterocolitis, significantly reduce the quality of life for many people with HSCR even after surgery. Due to these problems, a novel treatment, whereby stem cells are transplanted into the aganglionic segment to replace the missing ENS, has been proposed and over the last 10–15 years numerous international groups have been, and are currently, involved in preclinical studies aimed at developing such a cell replacement therapy.

To put this work in context, it is important to know that the human ENS contains approximately 500 million neurons and four times as many glia distributed along the entire bowel in two interconnected layers called the submucosal and myenteric plexus (Furness, 2006). These neurons and glia control bowel motility, respond to sensory stimuli, regulate blood flow, support epithelial function and modulate local immunity (Furness, 2012). To perform these roles, there are at least 14 enteric neuron subtypes (Furness, 2000) that express every neurotransmitter in the CNS and there are several types of enteric glia. All cells of the ENS are neural crest-derived and migrate into the bowel during week three to eight of human gestation (or day 9.5–13.5 of mouse fetal development) and then must differentiate and establish a sophisticated regulatory network (Sasselli et al., 2012b). The goal of stem cell therapy is to repair or replace defective or missing enteric neurons and/or glia to improve bowel function.

At the fourth international meeting "Development of the enteric nervous system; cells, signals, genes and therapy" held in Rotterdam, The Netherlands (April 2015), a multidisciplinary group of basic scientists and clinicians, including surgeons, gastroenterologists, and pathologists, decided that a White Paper should be written to clearly set out methods and approaches to identify, isolate, purify, expand and optimize ENS stem cells and progenitors, transplant them into the bowel, and assess transplant success as a way to restore gut function. By reviewing published studies on ENS stem cell therapy, we identified specific areas to help direct future research, gaps in knowledge, and strategies to address these challenges, taking advantage of knowledge gained from central nervous system (CNS) stem cell biology. Although there are no "gold standard" approaches to isolate and propagate ENS stem cells, published studies delineate many methods commonly used in this field. This White Paper aims to form a consensus and provide the ENS and stem cell biology communities with protocols for working with ENS stem cells, for their transplantation into the bowel, and for their subsequent analysis.

Considering the steady advance and success of several preclinical trials in animal models, we now need to consider "first in man" studies of stem cell therapy for enteric neuropathies. Here we also discuss obstacles that must be overcome to move ENS stem cell therapy to the clinic. This includes a discussion of the "best" diseases to initially treat, the accompanying safety studies that will need to be performed, and an outline of what "first in man" studies should include. With the emergence of new techniques, including approaches to label stem cells for transplantation and new gene editing technology, we are optimistic that ENS stem cells, capable of reforming enteric neuronal networks, will be obtained more reproducibly and with higher efficiency in near future. Here we aim to define standard methodologies that can be adapted to provide the necessary safety, regulatory and good manufacturing practice protocols required for eventual clinical application.

1. What are the target diseases for stem cell transplantation?

Neurogastrointestinal diseases are congenital or acquired disorders that affect the GI tract focally or diffusely and may involve all enteric neurons or only a subpopulation. Etiologies for neurointestinal diseases include genetic, inflammatory, degenerative, or paraneoplastic processes. Given this complexity, one needs to consider the underlying defect and its etiology in choosing the most reasonable targets for cell transplantation in animal models and, ultimately, for human clinical trials. Here we describe the pathophysiology of several neurogastrointestinal diseases that represent promising targets for cell-based therapy.

1.1. Hirschsprung disease (HSCR)

HSCR results from failure of enteric neural crest-derived cells to complete colonization of the distal intestine during fetal development. The uncolonized distal bowel remains aganglionic and tonically contracted, causing functional obstruction. Short-segment HSCR, in which the rectosigmoid colon lacks ganglion cells, affects 80% of patients, while the remainder have more extensive aganglionosis proximal to the rectosigmoid. Current treatment involves surgical removal of the aganglionic segment, but functional outcome is variable and many patients suffer life-long complications (Conway et al., 2007; Laughlin et al., 2012; Ludman et al., 2002; Pini Prato et al., 2008; Tsuji et al., 1999). This may reflect dysfunction of the so-called "normo-ganglionic" segment (Di Lorenzo et al., 2000; Kohno et al., 2007), abnormal anal sphincter function, retention of aganglionic distal bowel, or the sequelae of proctectomy. Enteric neuronal stem/progenitor cell (ENSC) transplantation provides a potential therapy to replace absent ganglia. For this purpose, ENSCs have been successfully isolated from ganglionic and aganglionic bowel of human HSCR patients and expanded in culture. These cells migrate and differentiate into neurons and glia following transplantation into embryonic hindgut (Almond et al., 2007; Metzger et al., 2009b; Wilkinson et al., 2015). Furthermore, studies using murine ENSCs from embryonic and postnatal intestine showed that transplanted ENSCs differentiate into neurons with processes that project into the gut muscle and form functional, synaptic connections (Hotta et al., 2013).

Identifying the optimal source of ENSCs for transplantation is a priority (discussed in Section 3). For clinical application, autologous cells avoid the issue of immunologic rejection. HLA-matched human embryonic stem cells or patient-specific induced pluripotent stem (iPS) cells also represent potential sources, but driving them along the correct lineage to generate functional enteric neurons and, if necessary, "correcting" the inherited genetic mutation present in those cells, remain major challenges. Choosing the right animal model of

aganglionosis to test cell-based therapy is also important (discussed in Section 2). Models used to date include benzalkonium chloride (BAC)-induced aganglionosis (Pan et al., 2011; Wagner et al., 2014) as well as Endrb-deficient (Gariepy et al., 1996) and Sox 10-deficient (Martucciello et al., 2007) rodents. These studies indicate that grafted cells can survive and migrate in the absence of an endogenous ENS, but whether they generate functioning neuronal networks in a postnatal host remains unclear. Efforts have been made to improve cell labelling to track transplanted cells (Natarajan et al., 2014) and to optimize cell delivery methods, laying the foundation for clinical application. Ultimately, however, demonstrating that transplanted ENSCs can ameliorate the motility defect present in animals models of HSCR must be accomplished before considering human application.

1.2. Esophageal achalasia

Loss of nitric oxide synthase (NOS)-expressing nitrergic neurons causes enteric dysmotility, including esophageal achalasia, where insufficient nitrergic neurons at the lower esophageal sphincter (LES) impairs its ability to relax, leading to a functional obstruction, dysphagia, and regurgitation (Vaezi, 2013). Current treatments target the LES using pharmacological therapy with nitrates or calcium channel blockers, physical disruption by pneumatic dilation, or surgical division (myotomy). These approaches all have a risk of complications, failure of symptom resolution, or disease recurrence. The hypothesis that LES dysfunction results from unbalanced stimulation by cholinergic nerves led to the use of botulinum toxin, a potent inhibitor of acetylcholine release. While botulinum toxin provides transient symptom relief, it is not a long-term cure (Boeckxstaens, 2006). Theoretically, the most physiological approach to treatment of achalasia lies in restoring the inhibitory, mainly nitrergic, elements of the myenteric plexus, which could be accomplished by transplantation of neuronal precursors to replace the missing population. Achalasia has been proposed to be an ideal target for ENSC transplantation (Schafer et al., 2009) for a number of reasons: (1) the neural deficit is well defined, (2) the target area (LES) is localized and small, and (3) neural precursors can be delivered endoscopically. However, significant challenges remain, including identifying the best source of regenerative cells, developing methods to grow large numbers of ENSCs *in vitro* prior to transplantation, and establishing methods to direct neuronal phenotype toward NOS expression.

1.3. Gastroparesis

Gastroparesis, characterized by delayed emptying of food from the stomach, is diagnosed clinically, based on nuclear medicine imaging tests or breath testing. A stomach biopsy is not typically performed. Thus, although neuromuscular abnormalities have long been suspected in idiopathic and diabetic gastroparesis, this has only recently been confirmed. Loss of interstitial cells of Cajal (ICC) and decreased neuronal nitric oxide synthase (nNOS) expressing cells are the most commonly identified abnormalities in the stomach of people with gastroparesis (Grover et al., 2011). The effect of injecting neural stem cells into the pylorus to induce muscle relaxation and accelerate gastric emptying has been tested using nNOS-deficient mice that are an established model of gastroparesis. Although grafted cells survived only one week, transplanted mice demonstrated improved gastric emptying (Micci et al., 2005). While not all patients with gastroparesis have loss or dysfunction of nNOS-expressing cells, these results suggest that ENSC therapy may benefit those individuals that do. Unfortunately, there is not a clear clinical correlation between improved gastric emptying and symptoms in people with gastroparesis, as gastric accommodation and sensory function may also be altered, so an additional question is whether this therapy will enhance quality of life even if gastric emptying improves.

1.4. Hypertrophic pyloric stenosis

Infantile hypertrophic pyloric stenosis (IHPS) occurs in 1–3 per 1000 children, usually 1 month-old males, and is characterized by pyloric muscle hypertrophy, resulting in luminal occlusion that leads to projectile vomiting, weight loss, and dehydration (Mitchell and Risch, 1993; Peeters et al., 2012). Surgical division of the pyloric muscle (pyloromyotomy) is an effective treatment, but subjects an infant to abdominal surgery. IHPS is hypothesized to be due to a marked reduction in the number of inhibitory nNOS fibers in the hypertrophied muscle (Vanderwinden et al., 1992). If this mechanism is correct, then ENSC transplantation might be an effective therapy that could be delivered endoscopically and without surgery. In support of this hypothesis, mice with targeted disruption of the nNOS gene demonstrate enlarged stomachs and gastric outlet obstruction due to pyloric hypertrophy (Huang et al., 1993). In this model, transplanted neural stem cells produce nNOS and ameliorate the pyloric obstruction (Micci et al., 2005). Therefore, cell therapy for IHPS may be an achievable goal, although further analysis is needed to determine how ENSCs lead to functional improvement and if the improvement is sustained.

One important issue is that IHPS may be clinically and genetically heterogeneous. In fact, IHPS may be due to defects in ICC or smooth muscle components (Peeters et al., 2012). Environmental factors have also been proposed as potential causes, including erythromycin exposure (Honein et al., 1999), feeding practice (Krogh et al., 2012), and cholesterol levels (Feenstra et al., 2013). Proper patient selection is critical for cell therapy success, since IHPS due to reduced nNOS-expressing neurons is much more likely to respond to ENSC transplantation than IHPS due to a primary myopathy or ICC defect.

1.5. Chronic intestinal pseudo-obstruction (CIPO)

CIPO is a clinical diagnosis describing patients with symptoms of small bowel obstruction and dilated intestine in the absence of mechanical blockage or aganglionosis. Occurring in about 1 in 40,000 live-births (Vargas et al., 1988), CIPO is a functional motility disorder of the small intestine that can have a neuropathic or myopathic cause (Knowles et al., 2013; Mousa et al., 2002). Although the neuropathy can have a variety of causes, including developmental, metabolic, inflammatory, infectious, and paraneoplastic, the majority of cases in children are idiopathic and affect the intestine diffusely (Heneyke et al., 1999). In a small number of pediatric cases, inflammation in the ENS, referred to as enteric ganglionitis (usually affecting the myenteric plexus), has been observed with lymphocytic (De Giorgio et al., 2002) or eosinophilic (Schappi et al., 2003) infiltrates in the ganglia. Neuronal injury or degeneration leads to intestinal dysmotility. Apart from small series reporting use of immunomodulators to treat inflammation when this is the underlying cause (De Giorgio and Camilleri, 2004), current treatment is supportive, with the provision of enteral and parenteral nutrition, and surgery to decompress the intestine.

Few animal models of CIPO exist (Clarke et al., 2007; Fu et al., 2013; Puig et al., 2009). A transgenic mouse with enteric neuropathy, delayed gastrointestinal transit, and selective loss of nNOS-expressing neurons has been described (Wangler et al., 2014) as well as a model of disordered ENS network formation and intestinal dysmotility secondary to mutations in planar cell polarity genes (Sasselli et al., 2013). Generation of additional models of neuropathic CIPO would facilitate future studies. Cell therapy could potentially be useful for treating highly selected cases of CIPO in which an enteric neuropathy is causative and where ongoing injury to the ENS, such as from paraneoplastic antibodies or active inflammation, is controlled. Since CIPO is a broad clinical diagnosis, rather than a definitive pathologic condition, careful evaluation to define disease etiology prior to transplantation is necessary for successful human therapy. The diffuse nature of Involvement adds an additional level of complexity in regard to cell transplantation therapies.

1.6. Neurogenic constipation and age-related loss of enteric neurons

Neurogenic constipation is a poorly defined condition that affects adults and children (Longstreth et al., 2006). Common causes include spina bifida and spinal cord injury (both extrinsic to the intestinal tract) and idiopathic slow transit constipation (intrinsic to the colon). The etiologies of intrinsic neurogenic constipation remain largely unknown. There are contradictory neurotransmitter data in pediatric slow transit constipation (King et al., 2010), with similar controversies in the adult literature (Walters et al., 2010). It is believed that the constipation which commonly accompanies human ageing is a result of age-related enteric neuronal loss (Bernard et al., 2009; El-Salhy et al., 1999). Animal models exist that may be useful to test the efficacy of cell therapy for treating neurogenic constipation (Zarate and Spencer, 2011).

1.7. Chagas disease

Chagas disease is caused by the parasite *Trypanosoma cruzi*. The disease is endemic in South and Central America and causes $>15,000$ deaths annually (Clayton, 2010). Acute symptoms go largely unattended and the infection subsides without treatment. However, some patients develop chronic infection, leading to cardiomyopathy, mega-esophagus and megacolon (Koberle, 1968) thought to be caused by massive loss of enteric neurons in affected segments of the gut (da Silveira et al., 2007; Meneghelli, 2004; Jabari et al., 2014; Ribeiro et al., 1998). Although animal models exist for Chagasic ENS alterations (Jelicks, 2010; Nogueira-Paiva et al., 2014; Teixeira et al., 1983), the potential of ENSC transplantation has yet to be tested.

1.8. Other enteric neuropathies

Gastrointestinal dysmotility has been associated with a wide variety of putative alterations in the numbers, shapes, and subtypes of enteric neurons (Knowles et al., 2010). However, apart from the aforementioned conditions and a few other rare disorders (*e.g.*, neuronal intranuclear inclusion disease), pathogenic connections between histopathological or immunohistochemical findings and impaired motility remain largely speculative. In some instances, conditions touted as primary neuropathies based on subtle neuropathological findings (*e.g.*, megacystic microcolon hypoperistalsis syndrome, X-linked intestinal pseudo-obstruction) were later shown to be disorders of smooth muscle (Kapur et al., 2010; Wangler et al., 2014). Many studies have examined neurotransmitter expression in the colon of patients with slow transit constipation, analyzing levels of a wide array of neurotransmitters, including VIP, NPY, 5-HT, Substance P, NO, and many others. The results of these studies, summarized previously (De Giorgio and Camilleri, 2004; Knowles and Martin, 2000) are highly variable, making it difficult to arrive at any definitive conclusions regarding the role of abnormal neurochemical coding in the pathophysiology of this condition. Obtaining reliable data in these studies is often hampered by small numbers of subjects, heterogeneity of patients, lack of precise criteria for diagnosing types of slow transit constipation, inconsistencies in tissue source and fixation method, absence of normative data, and lack of a reliable approach to quantitatively measure neurotransmitter amount. Expansion of the potential array of target diseases for ENSC transplantation will depend on multidisciplinary studies of patients with conservative interpretation of enteric neuromuscular pathology to establish objective diagnostic criteria for new or controversial conditions.

1.9. Phenotyping and genotyping of enteric neuropathies

One favored approach for cell therapy for enteric neuropathies is to transplant stem cells isolated from a normo-ganglionic region of a patient's gut into the affected region thus avoiding immune rejection. However, in order to do this, it will be important to phenotypically characterize patients both clinically, to clearly define the disease type, as well as pathologically, to inform on the status of the ENS. Further, genetic characterization of patients will need to be performed to determine whether they have mutations in known genes, whether these mutations would potentially compromise the ability of ENS stem cells to reform an ENS, and if so whether the genetic defect could be rescued with gene manipulation prior to cell transplant. The importance of genotyping is also relevant if patient-derived iPS cells are to be used for cell replacement therapy.

However, genetic characterization of patients with enteric neuropathies could prove problematic, not only due to the genetic complexity of HSCR but also to the lack of genetic understanding of the majority of other enteric neuropathies in general. For example, based on familial occurrence, recurrence in siblings, and on the presence of many naturally occurring animal models with colonic aganglionosis, HSCR is considered to be an inherited disease. However, the mode of inheritance can be dominant with reduced penetrance, mostly found in non-syndromic familial HSCR cases, whereas in families with syndromic HSCR a recessive pattern of inheritance is often observed. The sporadic cases, which comprise the majority, are considered oligogenic or even polygenic (Amiel et al., 2008). Genetic dissection of HSCR has, to date, implicated mutations in at least 16 genes that can cause, or contribute to, the development of HSCR, with *RET* representing the major HSCR gene (Bergeron et al., 2013). Most of the other genes have been identified in rare (familial) syndromic HSCR cases (Amiel et al., 2008). In addition to the rare, coding mutations with large effects, common, non-coding variants with small effects have also been identified (Emison et al., 2010; Jiang et al., 2015).

While HSCR is the "best understood" enteric neuropathy, its genetics are indeed complex and much remains to be learned. Most other enteric neuropathies are arguably even less well characterized, particularly genetically.

1.10. Conclusion

Achieving success in initial clinical trials of ENSC transplantation requires that we choose the right target disease. While most animal studies have focused on HSCR, this represents a high hurdle for clinical transplantation given the often extensive loss of enteric neurons and glia, and the associated secondary microenvironmental changes that create a milieu that may not be permissive to transplanted cells. We need to improve the characterization of all intestinal neuropathies to enhance our understanding about what cell types are abnormal, to determine the extent of the abnormality along the length of the gastrointestinal tract, and to identify prognostic factors that might predict which patients could most benefit from cell therapy. Initial trials should target diseases with focal loss of specific neuronal subtypes, as in esophageal achalasia or possibly gastroparesis. Clinical improvement in those conditions would set the stage for significant advances in the field, including optimization of cell isolation and culture methods and improved strategies for cell engineering and delivery.

2. What are the most appropriate models for experimentation and treatment of gastrointestinal neuropathies?

For a stem/progenitor cell therapy for gastrointestinal neuropathies, most prominently Hirschsprung Disease (HSCR), a vital staging-post to a clinical solution is choice of models. What are examples of the "right kind" of cells for therapy, and what are appropriate models of the affected bowel? What models and assays can be used to test cell migration, differentiation, connectivity, and function? Are the models used relevant for embryonic or post-natal conditions and does this matter? How does the species being used influence the results in

stem cell transplantation? What is the fastest or least expensive way to answer specific questions? When should a "step-up" be made to a more clinically relevant (likely more expensive) models? What model systems should be used before human translational studies?

2.1. Cell, tissue and organ culture models for ENS formation

2.1.1. Models of cells with ENS-forming competence

Enteric neural crest-derived cells are or include by definition the "right kind" of cells to form ENS; they can be harvested from the embryonic and postnatal gut. (Enteric neural crest-derived cells include differentiated ENS cells such as neurons and glial cells, as well as undifferentiated cells including ENSCs. Distinguishing between ENS glial cells, undifferentiated cells and enteric neural stem cells (ENSCs) remains a challenge, especially for live cell sorting; see Section 4). For mice, expression of reporters under the control of ENS regulatory elements (Corpening et al., 2011; Shibata et al., 2010) facilitates isolation of live enteric neural crest-derived cells by fluorescence-activated cell sorting (FACS) (Hotta et al., 2013), but this is not applicable to similar cells from humans. Neural crest-derived cells can be isolated from dissociated human (and rodent) gastrointestinal tissue by FACS after immunolabelling for the cell surface protein p75 (Chalazonitis et al., 1997; Walters et al., 2010) or from avian gut using the HNK1 antibody (Rollo et al., 2015). The isolated neural crest-derived cells, or at least a sub-population thereof, can be propagated (although this seems limited) in culture and studied *in vitro* or transplanted into recipient bowel. These cells, particularly those of human origin, are excellent models to test the capacities of ENS replacement. The rodent cells are readily obtainable but the human patient-derived cells require a close relationship with a clinical department as well as having particular ethical requirements (Hagl et al., 2013b).

Mouse and human pluripotent progenitor cells (ES and iPS cells) can be differentiated into "ENS-like" cells (Chambers et al., 2013). These pluripotent progenitor cells have the capacity to proliferate limitlessly, and iPS cells can be patient-derived and are therefore autologous and immunologically ideal. Theoretically these are the perfect clinical model cells but at present, the ENS differentiation conditions are not optimized.

2.1.2. Models of the bowel with enteric neuropathy

For therapeutic use, some form of ENS-competent cells must be combined with the patient's affected bowel. What would be a model for this bowel? Recipient ganglionated gut tissue can of course be obtained from a variety of normal sources and can be colonized by ENSCs *in vivo* in the mouse (Hotta et al., 2013), but this is not a model for any clinical condition. Aganglionic gut can be used in cell/tissue combination assays as a model for *e.g.* HSCR colon. This can be isolated from wild type mouse, rat and avian embryos prior to colonisation by ENSCs (Allan and Newgreen, 1980; Newgreen and Hartley, 1995; Young et al., 1998). This model bowel is entirely devoid of NC-derived cells (*i.e. aneural*), so as well as being less mature as a tissue, it is not a perfect model for the HSCR patient's distal colon which is *aneuronal*: it lacks enteric neurons but possesses extrinsic nerve fibers and some NC-derived glia. Aneuronal distal gut can be obtained from various mutant rodents (*e.g. Ret* $^{-/-}$ mice) even at post-natal stages (where survival permits), and this is a preferred model of the patient colon, including overgrowth of extrinsic nerve fibres, as in the human.

Aneuronal distal gut can also be obtained from avian embryos after vagal NC ablation (Yntema and Hammond, 1954) or after intestinal transection (Meijers et al., 1989) performed *in ovo*. However there are differences in structure and maturity of the avian colon and its extrinsic innervation (*e.g.* from Nerve of Remak as well as via the pelvic plexus). These differences mean that it is useful for basic questions but less useful as a clinical model.

Aneuronal gut tissue may be obtained from post-natal human distal colon tissue available from resections for HSCR (Rollo et al., 2016). Obviously this is ideal in principle but problems, especially of extended-term survival and growth *ex vivo*, have not been fully worked out yet. These technical difficulties especially apply when they involve large explants of colon tissues, as would be required to model treatments of the colon of post-natal patients.

In regard to aneuronal colon models, a recent paper describes late (i.e. post-natal) enteric neurogenesis from Schwann cell precursors in the mouse (Uesaka et al., 2015). The importance of this population for supply of enteric neurons will need to be evaluated in other animal, and human, models.

Gut tissue that models less extreme neuropathologies can be obtained from relevant mouse mutants (see below). As well as these complete gut tissues, decellularized gut can be obtained by careful detergent extraction (Totonelli et al., 2012); this may be useful for testing the role of gut ECM in ENS formation in the absence of living mesodermal, endodermal and endothelial cells.

2.1.3. Cells with ENS-forming competence, and affected bowel: models that bring the two together

Co-culture systems that are simplest to perform combine aneuronal gut tissues *in vitro* with ENS-competent donor cells. *In vitro* cultures on a solid substrate lead to dissolution of gut 3D structure, so organotypic methods have been used for rodent and avian gut. Gut segments grown on or in a matrix (*e.g.* collagen gel) or in fluid medium (Natarajan et al., 1999), or supported only at each end (Hearn et al., 1999) have been used to preserve 3D tubular gut form. These offer acceptable culture for periods of 4–8 days, allowing ENS cell proliferation, migration and differentiation (Hearn et al., 1999; Wang et al., 2011). Gut can also be cultured for weeks as transverse slices with preservation of 3D architecture (Metzger et al., 2007). Differentiation and a degree of morphogenesis of the gut tissue occurs *in vitro* but the gut does not greatly increase in size as it would *in vivo*. These techniques are ideal for whole mount fluorescent (antibodies, EdU, *etc.*) imaging and for time-lapse recording using fluorescent reporters (Druckenbrod and Epstein, 2007; Nishiyama et al., 2012; Young et al., 2004), and support limited functional studies. These *in vitro* assays are compatible with cell and tissue combinations from different species (Almond et al., 2007) because of the absence of immune responses.

For treatments of infants and children, the bowel will be growing, and this can be modelled using systems that supply blood to the host tissue. The simplest technique that provides blood to host tissues for avians, is chorio-allantoic membrane (CAM) grafts (Allan and Newgreen, 1980), where extensive gut elongation occurs. *In ovo* transplants (Le Douarin and Teillet, 1973) and intracoelomic grafts (Nagy and Goldstein, 2006) are also useful, but they are technically more difficult; in most cases CAM grafts would answer the same questions. These avian-based grafts permit xenografting since they commence before the onset of immune surveillance. Unfortunately, mammalian gut growth in CAM grafts seems impaired, possibly because the larger and less deformable avian red blood cells (Windberger and Baskurt, 2007) have difficulty negotiating mammalian capillaries. Moreover the avian system models embryonic events whereas clinically appropriate models would need to replicate post-natal stages. For rodent tissues, renal capsule grafts (Cass et al., 1992; Young et al., 1998) are useful even for extended periods (months) in contrast to the short duration of the avian grafts (CAM <9 days, intracoelomic <3 weeks). Post-natal human tissues can also be supported in renal capsule grafts to mice with severe combined immunodeficiency (SCID). As well as histology and immunolabelling, neurophysiological tests such as contractility responses to electrical and chemical stimuli (Newgreen et al., 1980) can be applied to these long-term grafts but their usefulness for studies of coordinated gut motility is impaired because the

grafts become distorted over time, and time-lapse imaging is minimal because the more mature tissue is more opaque.

These *ex vivo* models provide base-line information on the clonal and population expansion abilities of ENSCs, their ability to populate colonic tissues and assemble into ganglia in the correct position, to differentiate into the many different ENS cell types and in the appropriate ratios, and to connect via neurites and establish at least some level of ENS function. Yet these are "hurdle requirements"; a failure of ENS self-organisation in these models would suggest success could not be attained in a clinical setting, but success with these models (and much has been attained already) is a long way from predicting clinical utility.

2.2. Whole animal models of ENS pathologies

Stem/progenitor cell therapy to treat human ENS pathology will inevitably involve trials with whole animal models. The ideal models should have phenotypes (and genotypes) that resemble the human conditions described in Section 1. Developmental stage-wise, they will need to resemble the post-natal human colon, when enteric neuropathies are typically diagnosed.

2.2.1. Rodent models

Most studies to date use animal models of HSCR (Burzynski et al., 2009; Zimmer and Puri, 2015). Loss-of-function mutations in *RET* are the most common cause of HSCR in humans but the disease genetics are complex, involving non-coding as well as coding sequences. Furthermore there are many other gene defects that predispose to HSCR (Amiel et al., 2008). Mouse models are available with spontaneous and engineered mutations in essentially all of the HSCR-associated genes (*e.g. Ret, GDNF, GFRa1, ET3, EdnrB, Sox10;* see JAX database for many types, reviewed by (Zimmer and Puri, 2015) and there is also the important rat *EdnrB* model (Ceccherini et al., 1995). These rodent models are an excellent mimic of human HSCR because of the shared gene defects, but there are some obvious differences. For example, *Ret*$^{+/-}$ mice (*e.g.* 129S/Sv-*Ret*^{tm1Cos}/J) are asymptomatic, whereas humans with familial *RET* mutations are affected but with incomplete penetrance, estimated at about 70% for males and 50% for females (Attie et al., 1995). However, titrating *Ret* expression in mice to about 30% results in human HSCR-like phenotype with incomplete but male-weighted penetrance (Uesaka et al., 2008). Differences in gene dosage should be borne in mind before predicting human responses on the basis of rodent results.

The genetic background of inbred rodents also has important effects on ENS morphogenesis and disease phenotypes (Dang et al., 2011; Walters et al., 2010). This observation suggests that in outbred human populations there may also be diverse and unpredictable responses to cell transplantation because of unknown genetic modifier effects. Even now, a reflection of this variability is that a technically flawless HSCR resection/anastomosis is not a reliable predictor of long term outcome. It will be necessary to demonstrate robustness of results in several mouse strains. However, the unpredictability based on the genetic unknowns of individual humans will gradually recede as Whole Genome Screening becomes economically feasible.

ENS repair for a severe enteric neuropathy like HSCR to a functionally adequate stage would require time after delivery of ENCDCs, and in post-natal humans this would be available by performing a variant of the Swenson colonostomy procedure as a surgical holding measure while the "new" ENS adapts in the retained indwelling distal colon, prior to final reparative anastomosis. Even if matched for donor and host strains (Hotta et al., 2013), the size and delicacy of neonatal mice render such surgical approaches extremely challenging (Zhao et al., 2009), and functional assays to judge progress, such as manometry, are not yet possible. In any case, post-natal survival due to the gut disease and/or other defects is typically short. We therefore recommend against reparative cell emplacement and surgery trials in neonatal mice, despite the appropriate mutants. Larger animal models, starting with rats (Stamp et al., 2015), will overcome this problem because intestinal stoma creation is possible and is tolerated for extended periods.

An alternative strategy is to use mice with ENS defects that are not fatal. For example, *Gdnf*$^{+/-}$ mice have hypoganglionosis throughout the gastrointestinal tract (Flynn et al., 2007; Gianino et al., 2003) with impaired colonic motility, poor muscle contractility, dilated colon and fecal retention (Shen et al., 2002). These mice have a normal life expectancy (unlike many HSCR models), so long-term outcomes following experimental procedures can be studied *in vivo*. Also, since endogenous ENS cells are present, interactions (inductions, connections) between transplanted cells and the host ENS may be studied. Most importantly, this line would permit analysis of intestinal motility many months after transplantation. The Gdnf-flox line (B6.129S1(Cg)-*Gdnf* $^{tm1.1Neas}$/J) is also readily available, but the relevance of this model to human disease is uncertain since technical challenges analyzing neuron density in human specimens make ascertainment of hypoganglionosis challenging.

Achalasia, an acquired loss of peristalsis in the esophagus and impaired opening of the esophageal sphincter, may result from enteric neuron degeneration, especially nitrergic neurons. The mouse genetic model, *Nos1*^{tm1Plh}/*Nos1*^{tm1Plh}, has very low (not zero) levels of nNOS (in brain) and a complex multi-organ phenotype, and is a more extensive disease than human achalasia (Huang et al., 1993). Not only is there impaired relaxation of the lower esophageal sphincter, but also of the pyloric sphincter (Mashimo and Goyal, 1999), ileum (Mang et al., 2002) and proximal colon (Anitha et al., 2008), and an absent recto-anal inhibitory reflex (Terauchi et al., 2005), and these mice also have gastroparesis, or impaired emptying of the stomach (Mashimo et al., 2000). These digestive dysmotilities all occur in humans although often separately. This model offers several sites along the gastrointestinal tract for transplantation. Embryonic mouse neural stem cells injected into the pylorus of *Nos*$^{-/-}$ mice has been reported to differentiate rapidly into Nos-expressing neurons and symptoms (Micci et al., 2005) of gastroparesis are also alleviated. This extraordinary result requires much further work to confirm it, and to ascertain the mode of functional effects.

The *Spry2*^{tm1Ayos}/*Spry2*^{tm1Ayos} mouse also shows functional oesophageal achalasia with dilated oesophagus, but with hyperganglionosis of the ENS (unlike human achalasia) (Taketomi et al., 2005). This is superficially similar to human MEN2B, but it is genetically different. Its usefulness as model for human disease treatment does not seem high at present.

Diabetes is associated with reduced expression of NOS1 neurons as seen in diabetic gastroparesis (Grover et al., 2011), particularly in female patients. In diabetic mice, comparable changes occur in the ENS, including loss of nitrergic neurons (Bagyanszki and Bodi, 2012). Mice fed a high-fat diet also develop type 2 diabetes as well as obesity, and intestinal dysfunction with lowered numbers of nNOS and VIP neurons in the duodenum (Stenkamp-Strahm et al., 2013). In view of the clinical importance of diabetes and obesity in human health, these mice are particularly important targets for clinically motivated studies aiming at stemming the loss of these cells, or replacing them.

Although numerous mouse mutants have been very informative about the role played by certain genes in ENS development, one also needs to bear in mind possible non-cell-autonomous roles of these genes, and how complexities in gene function could affect future cell therapies for enteric neuropathies. For example, arguably the best known ENS development gene, Ret, has been shown to be involved in ENS precursor cell survival, migration, proliferation and differentiation (reviewed in (Sasselli et al., 2012b)). However, in studies where wild-type ENS progenitors were transplanted into the vagal NCC pathway of Ret-deficient embryos, these wild-type cells were only able to colonize the proximal foregut, demonstrating a non-cell-autonomous

effect of the Ret mutation on "normal" enteric progenitors (Bogni et al., 2008). Clearly this could be important should ENS stem cells, or cells from other sources, be transplanted into HSCR patients that have a *RET* mutation. More studies need to be performed to determine the effects of mouse mutations not only on enteric NCCs specifically, but also on their local environment.

2.2.2. Avian and Fish models

Formation of the ENS in Aves is comparable to that in mammals, with differences in detail: the colonic SMP is colonised first (Burns and Douarin, 1998) unlike the MP-first sequence in mammals (McKeown et al., 2001), neuronal differentiation lags further behind the colonising wavefront than in mice (Conner et al., 2003; Nagy et al., 2012) and trans-mesenteric migration (Nishiyama et al., 2012) from midgut to colon does not occur. Despite the dearth of genetic ENS models in Aves, whole animal models of HSCR can be produced by microsurgical vagal NC ablation (Yntema and Hammond, 1954) or intestinal transection (Meijers et al., 1989) at early stages *in ovo* as well as by pharmacological endothelin signalling inhibitors (Gasc et al., 2015). The HSCR-like phenotype of NC ablation can be saved by NC replacement (Barlow et al., 2008), but this is early embryonic, and is therefore of little use as a model of any anticipated clinical approaches to human post-natally detected enteric neuropathies.

The Zebrafish is an amenable model system (Shepherd and Eisen, 2011) with ENS colonization similar to that of amniotes with some differences. ENCDCs do not migrate within the gut mesenchyme but as two parallel chains just outside the gut. Also the later ganglionation as in the mammalian and avian ENS does not occur. *Colorless,* a *Sox10* mutant, is a HSCR model (Dutton et al., 2001; Kelsh and Eisen, 2000), while *lessen* has ENS cells along the entire gut but at lower cell density, like hypoganglionosis (Pietsch et al., 2006). However their usefulness in the context of developing reparative procedures for human infants and children is limited.

Despite practical uselessness for modelling repair procedures, these models, avian and fish, have provided, and will continue to provide, vital information on ENS formation. In particular they may be among the quickest and most economical avenues for obtaining information on growth factor requirements for the induction of cells with ENS-forming capacities (Reichenbach et al., 2008; Simkin et al., 2013), and this would most likely be translatable to human iPS cells. Further, both avian and zebrafish are excellent model systems for high throughput reverse genetic screening (*e.g.* using morpholinos and CRISPR knockdown technologies) of candidate genes implicated in ENS formation and for chemical screening of compounds that may affect enteric NCC migration, proliferation and/or differentiation *in vivo*. Although CRISPR technology is still in its infancy, particularly in the chick, chemical screening has recently been performed using zebrafish and chick to test the idea that certain medicines, taken during early human pregnancy, might alter HSCR risk (Schill et al., 2016).

2.2.3. Porcine models

Despite the genetic and descriptive similarities, the above models differ vastly from human neonates in the size of the field of recolonization required, so large animal models, despite the expense, will be necessary for ENS stem cell therapy proof-of-principle. For this pigs offer the advantages of similarity to humans in size, anatomy, physiology, and genetic makeup (Sri Paran et al., 2009). The pig is already a model for human physiology and a surgical model, with considerable ENS data (Barbiers et al., 1994; Brown and Timmermans, 2004; Montedonico et al., 2006). Pigs tolerate intestinal surgery and stoma creation with fortitude (J.B. Furness, personal comm.). We are not aware of porcine ENS pathology models, but the BAC process (see Section 1, p5 and below) could be employed to induce localised aganglionosis or hypoganglionosis to produce models of human enteric neuropathies. Potentially transplantable porcine ENS cells can be isolated by p75-FACS (B. N. Rollo, personal comm.). Autologous implanted cells would be required for cell survival past 1–2 weeks post-implantation, unless using immunodeficient pigs. However immunosuppression is achievable in pigs with a cocktail of drugs (Gruessner et al., 1996). Additionally, there are pig models with severe combined immunodeficiency (SCID) including SCID Yorkshire pigs (Basel et al., 2012), and the SCID Göttingen minipig (Lee et al., 2014). SCID pigs are available in U.S.A. from the National Swine Resource and Research Center (http://nsrrc.missouri.edu). The Goettingen minipig model has real advantages for adult studies because commercial adult pigs are large and cost more to feed and house, and are more difficult to handle. However, for paediatric surgery trials which would require only a limited survival time, the minipig does not offer significant advantages to offset their generally greater cost per weanling unit, availability and supply.

2.2.4. Chemically-induced models

Chemically induced aganglionosis, by topical application *in vivo* of BAC to small or large bowel, can result in local aganglionosis/hypoganglionosis (Pan et al., 2011; Wagner et al., 2014). This is not ENS-specific at the outset, but mesodermal cells are less affected/recover better from BAC, than the ENS. Aganglionosis/hypoganglionosis in the BAC model might not be permanent and neurons re-populate the lesioned area in time (Hanani et al., 2003).

2.3. Conclusions

Much information on ENS formation and on potential therapies for ENS diseases will continue to be gathered from *in vitro* models of varying types, in particular on comparison of the complexity of the distribution and wiring pattern and of the spectrum of neuronal differentiation in "replacement ENS" versus normal ENS. In addition detailed functional data will soon emerge from mouse whole animal models on, for example, control of smooth muscle function by replacement ENS. These extensions of current research using rodent, avian and fish can be anticipated in the near future.

There seem to be two next steps of equal importance. The first is a shift to the rat: much of the experience gained with the mouse will be applicable to this genetically convenient model, with the additional advantage that anticipated surgical approaches – colostomy, transfer of cells to the distal colon, and later bowel anastomosis – required for human HSCR postnatally are possible in the *EdnrB* rat HSCR model (Stamp et al., 2015). The second is to approach the potential scaling difficulty. The favourable results in the mouse suggest that transplantation therapies for ENS diseases will be possible in humans in principle, but all events of ENSC replacement for an ENS disease i.e. cell proliferation, migration, aggregation, differentiation, and wiring, must be done on a vastly larger scale in human patients than in mouse or even rat. The sheer size difference may lead to failure in clinical practice. It is imperative that a step to a human-scale model, the pig, be made, and this should be commenced sooner rather than later.

3. What is the optimal source of stem cells for enteric neuronal replacement?

During embryogenesis, neural crest progenitors colonise the developing bowel and ultimately give rise to all the neurons and glia of the ENS. However, the optimal source of stem/progenitors cells to generate a "new" ENS in the defective portion of bowel of patients with enteric neuropathies has yet to be established. Here we identify the main candidate stem/progenitor cell populations and highlight the respective pros and cons of each cell type in the context of enteric neuronal replacement. Of note is an exciting study recently published

by Studer and colleagues (Fattahi et al., 2016). These authors directed human embryonic stem (ES) cells towards a vagal neural crest (i.e. ENS precursor) lineage and showed that these ES-derived cells, when transplanted into the vagal neural crest region of developing chick embryos and into the cecum of young mice, migrated to the chick bowel and colonized the entire mouse colon respectively. *In vitro*, they enhanced smooth muscle differentiation, and differentiated into enteric neuronal and glial cells. Perhaps most interestingly from a cell therapy point of view, when the cells were transplanted into the cecum of *Ednrb*$^{-/-}$ mice, which have megacolon and usually die in early post-natal stages, all mice survived, and had transplanted cells along the colon, implying rescue of the aganglionic gut phenotype. These studies, for the first time, outline an efficient strategy to derive and purify enteric precursors from human ES cells that could potentially enable the large-scale production of specific human enteric neurons for cell therapy on demand (Fattahi et al., 2016). Nevertheless, before this becomes a reality some issues remain to be addressed such as the effect of transplanted cells on bowel motility, and long-term safety (Heuckeroth, 2016).

3.1. Enteric nervous system neural stem/progenitor cells

It has been established in rodents and in humans that resident ENS neural stem/progenitor cells exist in the GI tract in the postnatal period (Bixby et al., 2002; Bondurand et al., 2003; Kruger et al., 2002). HSCR is the result of an absence of the ENS in the distal bowel, however the remaining ganglionated bowel contains a mostly normally functioning ENS and likely the same resident neural stem/progenitor cells found in normal bowel. This offers the enticing possibility of using patient-derived, autologous neural stem/progenitors cells isolated from the normo-ganglionated regions of HSCR patient bowel as the source of cells to ultimately transplant and treat the disease (Rollo et al., 2016). There are several likely major benefits of using ENS neural stem cells to treat enteric neuropathies. These include:

(i) ENS neural stem cells have received the appropriate prior “education” to become enteric neural stem/progenitor cells. ENS-derived neural stem cells have arisen from the original source, chiefly the vagal neural crest. They have received the appropriate signals throughout development to become enteric neural crest cells, expressed the appropriate genes and have likely already given rise to functional mature neuronal daughter cells. A first step of this may be the acquisition of a vagal HOX code (Kam and Lui, 2015). A previous study showed that even vagal neural crest cells are not efficient at generating enteric neurons if they do not pass through their normal migratory route and receive specific cues, including retinoic acid signalling (Simkin et al., 2013).

(ii) Patient-derived autologous cells would avoid the need for immune suppression after transplantation. Stem cells from the normal regions of patient’s bowel can be expanded in vitro and transplanted back into defective regions of bowel of the same patient.

(iii) Gut derived ENS stem cells have proven ability to generate enteric neurons. Resident enteric neural stem cells have likely already given rise to functional neuronal daughter cells during generation of the ENS. Further, recent studies in mice have demonstrated that ENS-derived neural progenitors can give rise to neurons of the appropriate neurochemical and electrophysiological phenotype following transplantation into the postnatal colon (Hotta et al., 2013).

Caveats to the use of ENS-derived progenitor cells for cell therapy include:

(i) Gut derived ENS progenitors have limited capacity for self-renewal (Bondurand et al., 2003; Kruger et al., 2002) and large numbers of cells will be required to colonise even modest regions of aganglionic bowel. Therefore if in vitro expansion of these cells is limited, this could prove to be a major hurdle.

(ii) Gut derived ENS progenitors are difficult to purify. We currently lack robust cell surface markers for prospective isolation of enteric neural crest stem cells from the post-natal bowel. Recent studies in rodents have employed fluorescent transgenes from promoters of genes that encode proteins that are not expressed on the cell surface, so using these genetic markers for cell sorting in humans is not simple (Corpening et al., 2011).

(iii) Patient-derived ENS progenitor cells will possess genetic mutations that caused the disease we need to treat. This may not be a problem for most children with Hirschsprung disease where the proximal bowel ENS usually works well, but might be problematic for some types of neuropathic chronic intestinal pseudoobstruction syndrome (CIPO) (*e.g.*, POLG mutation, TYMP mutation). For these problems, in vitro manipulation or correction of the genetic defects using CRISPR/Cas9 or TALEN technology might be required, but this raises safety questions inherent to gene therapy (i.e., neoantigens and malignant transformation).

3.2. Non-ENS neural stem/progenitor cells

There are numerous other potential non-ENS sources of neural stem cells which could conceivably be used for cell therapy for enteric neuropathies. Here we discuss the pros and cons of each potential non-ENS source of cells for treatment of enteric neuropathies.

3.3. Central nervous system (CNS) neural stem cells

CNS neural stem cells can effectively treat numerous CNS neuropathies (Barker et al., 2013). Further, studies have shown that CNS-derived neural stem cells can survive and make contributions to functional improvements in gut motility disorders (Kulkarni et al., 2011; Micci et al., 2005, 2001). The benefits of CNS neural stem cells include:

(i) Close developmental association of the CNS/ENS. The vagal neural crest which forms the majority of the ENS arises from the neural tube adjacent to the developing caudal hindbrain and there are close similarities in the range of neurotransmitters expressed by CNS and ENS cells.

(ii) CNS neural stem cells are a well characterised population, whose culture conditions are well established and which possess an extensive capacity for self-renewal (particularly fetal derived neural stem cells).

Caveats for the use of CNS neural stem cells include:

(i) CNS-derived stem cells are not easily accessible since isolation involves highly invasive procedures, particularly accessing stem cell rich regions of the brain. Therefore, use of CNS neural stem cells for treatment of enteric neuropathies is unlikely.

(ii) CNS-derived stem cells may not be as efficient at migration and neuronal differentiation in the gut as ENS neural progenitors (Findlay et al., 2014).

3.4. Pluripotent stem cells

Both human embryonic stem (ES) cells and induced pluripotent stem (iPS) cells have the capacity to give rise to any cell of the body, including those of the ENS. Therefore, there is great interest

in the potential use of these pluripotent stem cell populations to treat enteric neuropathies (Heuckeroth, 2016). Potential benefits include:

(i) ES and iPS cells have a near unlimited capacity for self-renewal. This means that large numbers of cells likely to be required for ENS cell therapy can be produced from these pluripotent populations.
(ii) ES and iPS cells can be manipulated in vitro to induce neural crest-like phenotype (Chambers et al., 2009; Denham et al., 2015; Hotta et al., 2009; Lee et al., 2010, 2007) and the subsequent engraftment or coculture with embryonic gut tissue (chick or mouse) can induce enteric neuron-like cells (Denham et al., 2015; Hotta et al., 2009; Sasselli et al., 2012a). Recently, as mentioned above, protocols to obtain large numbers of ENS neural progenitors, that colonised the gut of Ednrb mutant mice following transplantation, have been published (Fattahi et al., 2016).
(iii) iPS cells (in contrast to ES cells) can be used for autologous transplantation; a patient's own skin or blood sample could be used to generate the iPS cell line that is used for transplantation.
(iv) ES cells and iPS are easily genetically manipulated. This may be important because some enteric neuropathies (*e.g.* HSCR, some types of CIPO) result from identifiable genetic changes that may need to be addressed before transplantation. iPS cells are easily genetically manipulated using CRISPR/Cas9, TALENs or other traditional gene manipulation techniques. However off-target effects must be considered when assessing safety for cell transplantation.

Caveats for using ES and iPS derived cells to treat enteric neuropathies.

(i) Ethical issues arise because ES cells are derived from human embryos. This problem does not occur with iPS cells that can be produced from readily available post-natal cells with little risk.
(ii) Transplantating ES- or iPS-derived cells raises safety concerns, especially the possibility of tranplantating residual pluripotent stem cells which may be tumorigenic under certain conditions.
(iii) Recapitulating embryogenesis in a dish, to generate enteric neurons from pluripotent stem cells, is difficult, costly and often inefficient using current techniques.

3.5. Other neural crest-derived stem cells sources

Numerous other sources of neural crest-derived stem cells reside in somatic tissues and could potentially be used for enteric neuronal replacement. These include sciatic nerve (Bixby et al., 2002), hair follicle bulge (Sieber-Blum et al., 2004), and dental pulp (Gronthos et al., 2000; Stevens et al., 2008). Although these populations are neural crest derived, they arise from varying axial levels of the neural tube, may have inappropriate epigenetic memory, and it is not known if these cells are capable of generating enteric neurons and glia.

It is currently difficult to define the optimal source of cells for enteric neuronal replacement. Each potential population of cells has their own benefits and caveats that need to be exploited and overcome, respectively, to prove efficacious for enteric neuronal replacement. Until such time, studies should not be limited to any one population of cells, as information gleaned from each study will benefit the field as a whole.

4. Identifying, selecting, harvesting and optimizing isolation of gut-derived ENS progenitors/stem cells

Enteric neural progenitors, which appear to reside within the ganglionated myenteric and submucous plexus of the gastrointestinal tract, can be isolated from the gut by dissection, using specialized culture conditions or by cell sorting. Techniques to isolate and culture the myenteric (Jessen et al., 1978, 1983; Korman et al., 1988; Nishi and Willard, 1985) or submucosal plexus (Surprenant, 1984) were developed in the late 1970s, and were even used for transplantation into the CNS (Tew et al., 1994). Unfortunately, most techniques isolated many non-ENS cells along with the ENS and the stem cells were only a small subset of the ENS cells isolated. Only Jessen's approach of manually dissecting the colonic myenteric plexus from guinea-pigs prior to dissociation delivered isolated pure ENS cells in small quantities. All these strategies have now been adapted for the isolation of enteric neural progenitors, and have been used, with variations, by different groups.

The most common approaches for isolating fetal and post-natal enteric neurons and glia, and their progenitors, begin with enzymatic dissociation of the bowel using dispase and collagenase followed by cell culture (Bondurand et al., 2003; Metzger et al., 2009a, 2009b; Schafer et al., 2003). Although the isolated cells are enriched for the ENS, cultures typically include fibroblasts, smooth muscle and immune cells in undefined combinations that could influence culture results or transplantation success. Indeed culture conditions may also dramatically alter cell composition with some cell types proliferating more than others. For example, some methods use FGF, EGF and chicken embryo extract, whereas others use only defined growth factor combinations. A detailed analysis of the amount of neural stem cells and their differentiation potential is necessary to allow comparisons between the different protocols. This is one of the basic tasks that have to be performed to allow a standardization of the "production" of neural crest derived stem cell for cell therapies. Depending on the reason for transplantation, the differentiation of specific subpopulations might be beneficial. So the influence of individual growth or neurotrophic factors upon the differentiation outcome is crucial, but the appropriate knowledge is yet to be provided.

One way to improve the purity of ENS progenitor cultures is by cell sorting. For this purpose, FACS appears to provide better defined cell populations than bead-based immunoselection. However, cell sorting necessitates very specific cell surface markers to identify progenitors within the enteric cell population or the use of transgenic mouse models where neural crest cells or progenitors are genetically labelled (*e.g.* Nestin, Wnt, Sox2). Numerous sorting attempts have been performed using antibodies against HNK-1 (aka NC-1) (Pomeranz et al., 1993), p75 (Chalazonitis et al., 1998; Wilkinson et al., 2015), integrin α4 (Bixby et al., 2002) or CD49 (Joseph et al., 2011), but when used individually they may fail to isolate the entire neural progenitor population. Whether this is of importance for transplantation success has yet to be determined as it is not yet known whether pure, well defined cell populations are better at rescuing the ENS than mixed cell populations.

One major problem for advancing stem cell therapy is isolating and obtaining sufficient numbers of ENS cells, particularly from post-natal bowel. Early protocols for the isolation of human myenteric plexus for example, yielded only single ganglia from postnatal gut (Schafer and Mestres, 1997), but more recent protocols using purified collagenase allow the isolation of pure myenteric plexus from human gut in larger quantities (Grundmann et al., 2015). Isolation of human submucosal plexus is also possible, but does not deliver coherent networks, as seen for the myenteric plexus. Moreover, there are plenty of neural stem cells in between the smooth muscle cells. These cells can only be isolated with specific markers for cell sorting. Whether the individual stem cell populations in myenteric, submucous and muscle layers are equal has to be analysed in more detail.

4.1. Optimizing, propagating, and priming stem cells prior to transplantation

To date, it remains unclear how many cells are necessary to colonize a defined area of aganglionic gut. Depending on the methods of cultivation and transplantation, as well as possible genetic or chemical modifications, the necessary number may vary significantly. In humans, the amount of available tissue for the isolation of enteric neural progenitors is restricted and only small numbers of neural stem cells might be available. It is therefore crucial to develop techniques to increase the number of ENS progenitors in culture prior to transplantation. Progenitor numbers can be expanded in culture by using mitogens such as GDNF, FGF and EGF. In addition, factors that have been effective for CNS-derived neural stem cells might also be applicable for the ENS (*e.g.* LIF, Interleukins, *etc.*). Recently it was demonstrated that granulocyte-colony-stimulating factor (GCSF) can significantly increase the amount and size of enteric neurospheres (Schuster et al., 2014b). Bacterial lipopolysaccharides, which seem to maintain the stemness of the enteric neural progenitors (Schuster et al., 2014a) may also be used to enhance the proliferation of enteric neural progenitors. Other important factors include Endothelin-3 which seems to maintain the multilineage potential of ENS precursors. This is a very important aspect, due to the fact that neural progenitors appear to lose their stem cell characteristics in long term cultures (Lindley et al., 2009).

Neural progenitors are often cultured as neurospheres, but could likewise be kept in adhesion cultures, or in a combined sphere-adhesion culture, grown on polystyrol beads. Moreover, the specific culture conditions, such as mimicking realistic oxygen concentrations (Hegewald et al., 2011; Mohyeldin et al., 2010), might also be an option to increase the yield of enteric progenitor cells since Hegewald et al. showed that the amount of p75-positive cells in culture increased when oxygen tension was reduced. Reduced oxygen tension might even be used to enhance the specific differentiation of neuronal subtypes. In CNS-derived stem cells, a reduction of the ambient oxygen tension to 3% led to a significant increase in the yield of dopaminergic neurons (Krabbe et al., 2009).

To increase the developmental potential of the cells for transplant, a pretreatment with neurotrophic factors or genetic modification strategies could be applied, as has already been demonstrated for cell therapies in the CNS (Fjord-Larsen et al., 2005). While genetic modification might be harder to control, a chemical priming with neurotrophic factors prior to transplant, as well as the use of specific devices (*e.g.* lipid nanocarriers) for controlled drug release might be advantageous.

4.2. Conclusions

We are still at the stage where the advantages and disadvantages of various approaches for the isolation, expansion and optimisation of neural progenitors/stem cells from the human ENS need to be investigated more intensely. At the current time there is no optimal and standardized way of isolating and expanding ENS progenitors while maintaining their stem cell properties. To address this, the field will need to develop culture conditions that mimic, more realistically, the neural stem cell niche of the ENS. Possible underexplored approaches include using hydrogels for cell cultivation that can be modified and tailor made to provide *in vivo*-equivalent conditions. Including mesenchymal stem cells, myofibroblasts, or even the gut microbiota might help to maintain such a niche allowing in vitro expansion, but growing cells under these conditions would necessitate development of additional ENS precursor isolation procedures prior to transplantation. There is also a need to develop cryopreservation strategies to permit storage of enteric neural progenitors, while retaining their characteristic features necessary for engraftment and colonization after transplantation into gut.

5. How are “neurospheres”, and the neural progenitors within them, best characterized?

Historically, the first characterization of neural stem cells derived from the central nervous system (CNS) dates back to the discovery of adult neurogenesis by Altman (1969). However, it was not until 1992 that cells with stem-cell potential were cultured in vitro to form the free floating three-dimensional spheroids that became known as neurospheres (Reynolds et al., 1992). Twenty years later several groups adapted protocols for the isolation and characterization of neurospheres from both rodent and human gut, which appeared to be very similar to their CNS-derived counterparts (Bondurand et al., 2003; Kruger et al., 2002; Rauch et al., 2006; Schafer et al., 2003).

5.1. Characterisation of neurosphere-like bodies (NLBs) from animal models

To assess the different cell types in a neurosphere, otherwise known as a neurosphere-like body (NLB), it is critical to analyze different molecular markers, gene expression and/or biological functions that distinctly characterize each cell population (progenitor, neuron, glia cell, myofibroblast, others; see Table 1 and Schweitzer et al. (2005) and Anderson (1983)). Depending on the individual culture protocols and markers used, NLBs from early postnatal mouse/rat gut are immunoreactive for typical neural differentiation markers after an *in vitro* culture period up to 2 weeks (Belkind-Gerson et al., 2013; Binder et al., 2015; Bondurand et al., 2003; Dettmann et al., 2014; Kruger et al., 2002; Silva et al., 2008). A small fraction of NLB cells ($<15\%$) remain negative for all differentiation markers used, raising the possibility that at least a subfraction of the isolated cells that generate neurospheres are undifferentiated progenitors or NSCs (Bondurand et al., 2003). This assumption is supported by studies demonstrating $\sim$15–30% proliferative cells within postnatal murine neurospheres after short-term BrdU or EdU pulse labeling (Mohr et al., 2013; Theocharatos et al., 2013) will co-labeled with neural markers (TuJ1, NOS, GFAP, Sox10, S100b) after a 96 h chase. After an extended culture period under differentiation conditions around 65% of neurons were co-immunostained with BrdU underlining that differentiated neurons are indeed derived from undifferentiated progenitors (Almond et al., 2007; Dettmann et al., 2014).

However, to date no definitive molecular marker of NSCs in both the CNS and ENS has been found. None of the markers currently used detects all progenitor subtypes at any given point of time or region in development. Although some surface and intracellular markers are highly expressed in enteric progenitors during embryonic gut development of rodents (i.e. especially Sox10, Sox2, RET, p75, Phox2b, EDNRB, Mash1, Nestin; reviewed in (Obermayr et al., 2013; Sasselli et al., 2012b) most of these markers are also expressed in differentiated neural cells (*i.e.* the majority of postnatal ENS cells) or other cell types and therefore discrimination of progenitors is not possible. Under standard culture conditions about 20% of initial cell colonies are immunoreactive for the neurotrophin receptor p75 after 7 days *in vitro*. If hypoxic culture conditions are used this potential stem and progenitor cell fraction can be doubled within the same time (Hegewald et al., 2011). However, it is very likely that truly undifferentiated cells (i.e., NSCs that can fulfill the operational stem cell definition in vitro and, most importantly, *in vivo*) represent only a small percentage of the cells within the neurospheres (presumably less than 2% based on earlier CNS studies (Gritti et al., 1996). Interestingly, one study of postnatal rat neurospheres identified typical markers of pluripotent cells such as Sox2, Nanog and Oct4 in a relatively large subfraction of cells even though most cells in NLBs are unlikely to have significant stem cell like properties (Hagl et al., 2013b). It will be interesting

Table 1
Molecular markers expressed in proliferating and differentiated enteric neural cells/progenitors/neurospheres *in vitro*.

Stem/progenitor marker	Recognition	Further cell detection	Species (M, H, R, C)	References
Ret	Receptor tyrosine kinase	Neurons	M, H	Bondurand et al., 2003; Metzger et al., 2009a; Sribudiani et al., 2011
p75	Low-affinity neurotrophin receptor	Glia Neurons	M, H, R	Binder et al., 2015; Bixby et al., 2002; Hegewald et al., 2011; Hetz et al., 2014; Kruger et al., 2002; Lindley et al., 2009; Lo and Anderson, 1995; Metzger et al., 2009a, 2009b; Mohr et al., 2013; Sribudiani et al., 2011; Theocharatos et al., 2013
Nestin	Intermediate filament type VI	Neurons Glia Other	M, H, R	Binder et al., 2015; Grundmann et al., 2015; Hagl et al., 2013b; Hetz et al., 2014; Metzger et al., 2009a, 2009b, 2007
HNK-1-NCAM (CD 57)	Integral membrane form of N-CAM (neural cell adhesion molecule)	Neuroepithelial cells	M,R, C	Schafer et al., 2003
Sox2	SRY (sex determining region-Y) HMG box 2	Glia	M, H	Heanue and Pachnis, 2011; Hetz et al., 2014
Sox10	SRY (sex determining region-Y) HMG box 10	Glia	M, H	Binder et al., 2015; Bondurand et al., 2003; Metzger et al., 2009b
Ki-67	Nuclear protein associated with ribosomal RNA transcription	Neural progenitors Glia	M, H	Binder et al., 2015; Hegewald et al., 2011; Metzger et al., 2009b
BrdU, EdU	Thymidine analogs	Neural progenitors Glia	M, H	Dettmann et al., 2014; Hegewald et al., 2011; Hetz et al., 2014; Metzger et al., 2009a, 2009b, 2007; Mohr et al., 2013; Theocharatos et al., 2013
pH3	Phospho-Histone 3	Neural progenitors Glia	M	Binder et al., 2015

Neuronal marker	Recognition	Further cell detection	Species (M, H, R, C)	References
TuJ1	Neuron-specific beta tubulin III		M, H, R	Binder et al., 2015; Bondurand et al., 2003; Hagl et al., 2013b; Hegewald et al., 2011; Hetz et al., 2014; Metzger et al., 2009a, 2009b, 2007; Mohr et al., 2013; Suarez-Rodriguez and Belkind-Gerson, 2004; Theocharatos et al., 2013
PGP9.5	Neuron-specific 27-kDa intracellular C-terminal ubiquitinilated hydrolase		M, H	Bondurand et al., 2003; Dettmann et al., 2014; Hegewald et al., 2011; Lindley et al., 2009; Metzger et al., 2009a, 2009b; Rauch et al., 2006
HuC/D	RNA-binding protein		M, H	Dettmann et al., 2014; Hetz et al., 2014; Mohr et al., 2013
Peripherin	Type III Intermediate filament		R	Bixby et al., 2002; Kruger et al., 2002
Mash1	Mammalian achaete–scute homologue 1		M	Bondurand et al., 2003
MAP2	Microtubule-associated protein 2		H	Metzger et al., 2009a
Neurofilament medium protein (NFm).	Intermediate filament protein		H	Metzger et al., 2009a
160/200-kDa NF	160- and 200-kDa proteins of human neurofilament		M	Suarez-Rodriguez and Belkind-Gerson, 2004
Tau	Microtubule-associated protein		M	Suarez-Rodriguez and Belkind-Gerson, 2004
VIP	Vasoactive intestinal peptide		M, H, R	Binder et al., 2015; Bondurand et al., 2003; Kruger et al., 2002; Metzger et al., 2009a, 2009b; Suarez-Rodriguez and Belkind-Gerson, 2004
nNOS	Neuronal nitric oxide synthase		M, H, R	Binder et al., 2015; Bixby et al., 2002; Dettmann et al., 2014; Hegewald et al., 2011; Kruger et al., 2002; Metzger et al., 2009a; Suarez-Rodriguez and Belkind-Gerson, 2004; Theocharatos et al., 2013
ChAT	Choline acetyl transferase		M, H	Hegewald et al., 2011; Metzger et al., 2009a, 2009b
AChE	Acetycholine esterase		M	Suarez-Rodriguez and Belkind-Gerson, 2004
CGRP	Calcitonin gene-related peptide		M, H	Binder et al., 2015; Metzger et al., 2009a, 2009b; Suarez-Rodriguez and Belkind-Gerson, 2004
NPY	Neuropeptide Y		M, H, R	Binder et al., 2015; Bixby et al., 2002; Bondurand et al., 2003; Kruger et al., 2002; Metzger et al., 2009a; Suarez-Rodriguez and Belkind-Gerson, 2004
Peptide YY	Agonist of the neuropeptide Y receptor		M	Suarez-Rodriguez and Belkind-Gerson, 2004
Peptide P	Neuromodulator and neurotransmitter		M	Suarez-Rodriguez and Belkind-Gerson, 2004
Galanin	Inhibits secretion of transmitters or hormones		M	Suarez-Rodriguez and Belkind-Gerson, 2004
TH	Tyrosine hydroxylase		M, H, R	Bondurand et al., 2003; Hagl et al., 2013b; Metzger et al., 2009a
Serotonin	Neurotransmitter		M, H, R	Kruger et al., 2002; Metzger et al., 2009a
DβH	Dopamine-beta-hydroxylase		R	Kruger et al., 2002
Glutamate transporter EACC1	EAAC1 glutamate transporter, sodium-dependent		M	Suarez-Rodriguez and Belkind-Gerson, 2004

Table 1 (*continued*)

	Recognition	Further cell detection	Species (M, H, R, C)	References
Synaptophysin	38-kDa glycoprotein of pre-synaptic vesicles		M	Suarez-Rodriguez and Belkind-Gerson, 2004
Glial marker	**Recognition**	**Further cell detection**	**Species (M, H, R, C)**	**References**
GFAP	Intermediate filament protein		M, H, R	Binder et al., 2015; Bixby et al., 2002; Bondurand et al., 2003; Dettmann et al., 2014; Hagl et al., 2013b; Hegewald et al., 2011; Kruger et al., 2002; Metzger et al., 2009a, 2009b, 2007; Mohr et al., 2013; Theocharatos et al., 2013
S100b	Calcium-binding protein B		M, H	Binder et al., 2015; Dettmann et al., 2014; Hetz et al., 2014; Lindley et al., 2009; Metzger et al., 2009a, 2009b; Theocharatos et al., 2013
Others	**Recognition**	**Further cell detection**	**Species (M, H, R, C)**	**References**
c-kit	Mast/stem cell growth factor receptor	Interstitial cells of Cajal (ICCs)	M	Binder et al., 2015
PDGFRalpha	Platelet-derived growth factor receptor, alpha polypeptide	Mesenchymal-derived cells	M	Binder et al., 2015
SMA	Smooth muscle actin	Myofibroblasts	M, H, R	Binder et al., 2015; Bixby et al., 2002; Bondurand et al., 2003; Dettmann et al., 2014; Hegewald et al., 2011; Hetz et al., 2014; Kruger et al., 2002; Metzger et al., 2009a, 2009b; Suarez-Rodriguez and Belkind-Gerson, 2004

to clarify the biological role of these genes in enteric neural precursor cells.

Some investigators have attempted to enrich putative fetal and postnatal ENS progenitors using either surface markers such as RET (Binder et al., 2015; Lo and Anderson, 1995; Natarajan et al., 1999), the neurotrophin receptor p75(NTR), α4 integrin (Bixby et al., 2002; Kruger et al., 2002; Mosher et al., 2007; Tsai et al., 2011) or Sox2/Sox10/Nestin promoter-driven reporter genes (Bondurand et al., 2006; Heanue and Pachnis, 2011). Using these approaches only some of the selected cells proliferated and not all proliferating colonies were equally multipotent. With respect to markers identifying differentiation, unpurified NLB cultures showed an increasing number of mature neurons and glia during *in vitro* culture and after about 2 weeks only 2% of cells remained negative for the pan-neural markers beta-tubulin III or the glial protein S100beta suggesting that the applied culture conditions support the *in vitro* differentiation of neurogenic and gliogenic progenitors (Binder et al., 2015). Further, qualitative immunostainings for neuronal subtypes could be demonstrated, which include markers for nitric oxide synthase (NOS1), choline acetyltransferase (CHAT), vasoactive intestinal polypeptide (VIP), neuropeptide Y (NPY), calcitonin gene-related peptide (CALCA or CGRP), tryptophan hydroxylase (TPH1) and tyrosine hydroxylase (TH); however especially TPH1+ (serotonergic) and TH+ (sympathoadrenal) neurons tend to decline with increasing age of donor tissue (Table 1; (Almond et al., 2007; Binder et al., 2015; Bondurand et al., 2003; Kruger et al., 2002).

5.2. Characterisation of human NLBs

More recently, protocols from rodent studies were adapted for human neurosphere propagation (Binder et al., 2015; Lindley et al., 2009; Metzger et al., 2009b; Rauch et al., 2006). As in rodents the extent of NLB expansion, size and differentiation potential of ENS precursors was dependent to the donor age. Molecular markers were adapted from rodent studies assuming similar cell populations can be identified. Thus, in primary human NLBs up to one third of cells were immunoreactive for p75(NTR) and a subfraction (not yet quantified) expressed the putative intracellular/nuclear progenitor markers Sox2, Sox10, Nestin, BrdU and Ki67 (Binder et al., 2015; Hetz et al., 2014; Lindley et al., 2009; Metzger et al., 2009b; Rauch et al., 2006). After multiple cell passages, the proportion of p75+ cells seemed to increase up to ~50% in secondary and ~60% in tertiary spheres. Again, as for rodent enteric neural crest-derived cells all currently applied 'progenitor' markers including p75 are also present in differentiated human neural cells making clear discrimination difficult. This is also true for Nestin, a known intracellular CNS progenitor marker, which was demonstrated in both neural and non-neural crest-derived cells (Binder et al., 2015; Rauch et al., 2006). Nevertheless, BrdU-uptake assays and subsequent co-labeling with TuJ1 and GFAP supports the idea of a small proliferating neural stem cell pool within the human spheres that can differentiate into neurons and glia (Metzger et al., 2009b). Furthermore, postnatal single-cell clonogenic cultures indicated an overall bipotential frequency of ~4% based on TuJ1 and S100 co-immunostainings after 10 days *in vitro* (Metzger et al., 2009b). Similar to their rodent counterparts, *in vitro* generated human NLBs contained a large fraction of differentiated cells indicated by PGP9.5 and S100 immunostainings (Lindley et al., 2009, Metzger, 2009a #2055). Whereas the relative PGP9.5 fraction remained constant (~50% of total cells), the S100 fraction seemed to decrease during multiple passages *in vitro* indicating a neuronal drift over time. Interestingly, PGP9.5 seems not only to be a pan-neuronal marker, but appears also in the early enteric neuronal progenitors (Rauch et al., 2006; Sidebotham et al., 2002). In the fetal human gut, all cells within premature ganglia are at the same time PGP9.5 and Nestin-positive, while in the late fetal gut only developing neurons remain PGP9.5 positive (Rauch et al., 2006). So far, only one study applied cell sorting strategies to enrich for human enteric neural (progenitor) cells (Binder et al., 2015). Following flow cytometry to select p75+ve human postnatal cells from submucosal biopsies subsequently generated NLBs contained almost exclusively neurons and glia (TuJ1 = 74%; S100=24%), but no smooth muscle positive cells. After induction of differentiation, a panel of characteristic neuronal subtype markers could be

demonstrated within postnatal neurosphere-derived colonies, which included CALCA, NOS1, serotonin, VIP, and CHAT (Metzger et al., 2009b). As in rodents, colonies from human adult donors may be more restricted in their differentiation potential, although there are some discrepancies between different studies depending on the individual culture conditions and analyses applied (Hagl et al., 2013a; Metzger et al., 2009a). More than half of the differentiated adult cells were immunoreactive for smooth muscle actin, about one third stained for either TuJ1 or S100. Interestingly, a subfraction of differentiated adult neuronal cells seem to express functional voltage-dependent sodium channels as shown via patch-clamp electrophysiology (Metzger et al., 2009a).

In summary, little is known about the relationship and between cells within gut-derived NLB and currently no exclusive stem cell marker has been identified. This highlights the need for a battery of markers and standardized approaches to be used to analyse the composition of NLBS and NLB-derived differentiated cell entities. The ability to purify enteric progenitor cells is not only essential for basic research but particularly for potential cell transplantation therapies aimed at rescuing or restoring the ENS in various diseases of the gastrointestinal tract.

5.3. What can the ENS field learn from the CNS field concerning neurosphere characterization?

Research in both the central and the enteric nervous system share methodological and biological features, and transferability of techniques and results might help identify limitations and problems that the relatively young ENS stem cell community is still confronted with. One important topic is the acknowledgement of common operational definitions, as they are a prerequisite for scientific cooperation and technology transfer in the field. Current studies of both CNS and ENS are not able to fully show all the formal requirements for true stem cells met by neurospheres (Pastrana et al., 2011). In the CNS field, this problem is recognized by calling neural stem cells (NSCs) "stem-like" or "reporter cells" (Lee et al., 2010; Park et al., 2006). A similar definition for ENS derived NLBs might prevent significant confusion. Further, because no exclusive marker for the isolation of neural stem cells exists, in the CNS field many groups therefore have used combinations of two or three markers or markers for negative selection (see Pastrana et al. (2011)). In future studies, it might be interesting to prove similar marker profiles also in the ENS or to apply broader age-dependent screening approaches aiming at identification of novel enteric stem cell (surface) marker genes. Interestingly, Parker et al., published a gene-expression profile comparing NSCs clones (showing clonal self-renewal and clonal multipotentiality) with those obtained from the neurosphere assay (Parker et al., 2005). The authors demonstrated that so called "stemness" genes expressed by both populations differed from a stem-like pattern in the operationally defined NSCs, towards a more differentiated one in the cells obtained from the assay. Moreover, it has been shown that neurosphere-NSCs gene expression is a dynamic process varying during culture time from a more undifferentiated to a more differentiated state (Gurok et al., 2004). Thus there is obviously a need for studies going beyond the neurosphere and to put greater effort towards the identification of definitive markers with both high specificity and selectivity.

Finally, to unravel the above mentioned influence of different culture conditions and sorting strategies on in vitro stem cell behavior it might be helpful to analyze enteric neural crest-derived cells using recently developed transgenic reporter mouse models to track cell cycle (Abe et al., 2013; Mort et al., 2014), stress responses (Thorp et al., 2011), chromosomal instability (Balbach and Boiani, 2015) or stem cell signaling (Ferrer-Vaquer et al., 2010) (Balaskas et al., 2012) *in vitro* and *in vivo*. Furthermore, embryonic development can be simulated *in vitro* using recent (human) pluripotent stem cell technologies to understand crucial molecular checkpoints for differentiation towards enteric neural progenitors and fully mature neural cells (Kawaguchi et al., 2010; Sasselli et al., 2012a). Clearly, much more fundamental research is needed before we can make significant progress towards a standardized protocol to mark, isolate and harvest enteric progenitors. The many parallels and previous successes in CNS stem cell research should encourage ENS researchers to further advance their scientific concepts.

6. What is the best way to deliver stem cells to the gut?

While the gut is a relatively accessible organ compared to the brain or spinal cord, delivering cells could be challenging given the size of the target organ. Moreover, its complex multi-layered organization, relatively loose connective tissue, and substantial venous and lymphatic flow could compromise or facilitate cell engraftment. Optimization of cell delivery is critically important to maximize cell engraftment if we hope to improve gut function, which is the ultimate goal of ENS stem cell transplantation for enteric neuropathies. To date, several approaches have been attempted to introduce cells into the gut wall of laboratory animals: (1) direct injection to the gut wall, (2) neurosphere implantation, (3) serosal application, (4) intraperitoneal injection, and (5) intravascular delivery. These methods have not yet been systematically investigated to determine which approach is best. Whatever approach is chosen will need to be minimally invasive, capable of delivering large numbers of donor cells, allow accurate targeting of cell delivery, and lead to effective cell spreading throughout the area of disease. In this section, we describe several delivery methods previously reported in the field of ENS cell therapy research. We also discuss approaches that have been employed in other areas of regenerative medicine and may have potential application in the intestine.

6.1. Injection of cell suspension into the gut wall

6.1.1. Seromuscular approach

Most studies to date have introduced neuronal precursor cells by laparotomy and direct injection into the gut wall (Anitha et al., 2008; Dong et al., 2008; Liu et al., 2007, 2013; Micci et al., 2005; Natarajan et al., 2014). This delivery method allows accurate targeting and permits introduction of large numbers of cells by multiple or large volume injections. Cells have been suspended at 50,000–400,000 cells per microliter and microinjected into the gut 2–4 places through pulled capillary glass needles or metal needles at 2–50 μL per injection (Anitha et al., 2008; Dong et al., 2008; Liu et al., 2007, 2013; Micci et al., 2005; Natarajan et al., 2014). Although some investigators have shown functional recovery of mice with enteric neuropathies following cell injection (Anitha et al., 2008; Micci et al., 2005), this method has several drawbacks. First, there is significant leakage of cells through puncture holes and the final location of these cells is not known. Second, the spreading of cells is poorly controlled, resulting in a random distribution and poor reproducibility. Finally, suspension of cells into delivery vehicle, after proteolytic dissociation of neurospheres can predispose to cell death following transplantation. Some of these drawbacks might be partially overcome by incorporation of cells into biomaterials such as hydrogels, which polymerize in situ and enhance retention (Lu et al., 2009), or by co-injection with a caspase-1 inhibitor to enhance cell survival (Micci et al., 2005). Despite these drawbacks, seromuscular cell injection has significant potential for clinical application as it can be used in large animals, including humans, and is amenable to minimally invasive laparoscopic surgical techniques which, combined with ultrasound, could allow accurate targeting into specific layers of the gut.

6.1.2. Intraluminal approach (Endoscopic approach)

In addition to trans-serosal injection of a cell suspension into the gut, cells can also be injected through the mucosa via endoscopy, a clinically relevant and minimally invasive delivery method. Endoscopy is a well accepted and commonly used technique in the diagnosis and treatment of gastrointestinal diseases in clinical medicine. Cheng and colleagues recently reported the use of colonoscopy to deliver enteric neuronal stem/progenitor cells into the aganglionic distal colon of mice with Hirschsprung disease (Cheng et al., 2015). Injection of a cell suspension (50,000 cells in 50 μL volume) was performed under direct visualization and cell spreading was observed within the submucosal layer extending circumferentially and for a longitudinal length of 1mm at 1 week following injection. One disadvantage of this method involves the technical difficulty and the (probably very small) risk of intestinal perforation during the procedure. However, endoscopic ultrasound can be added to facilitate more precise and safe delivery for future clinical application.

6.2. Implantation of neurospheres into the gut wall

During development, enteric neural crest cells migrate in chains as they colonise the gut, and the enteric neurons and glia in the mature gut reside in clusters known as ganglia (Druckenbrod and Epstein, 2005; Faure et al., 2007; Obermayr et al., 2013). These and other data indicate that enteric neural crest cells and their derivatives require high cell-cell contact for their survival and migration (Breau et al., 2006; Hackett-Jones et al., 2011). Generation of neurospheres *in vitro* from enteric neural progenitor cells provides a three-dimensional structure with high cell-cell contact that can be used for transplantation. Previous studies have shown proliferation, extensive migration, and appropriate neuronal and glial differentiation following transplantation of enteric neurospheres into the distal colon of wild type mice (Binder et al., 2015; Dettmann et al., 2014; Hotta et al., 2013).

The benefits of neurosphere implantation are demonstrated by the high rates of cell recovery, subsequent formation of ganglion-like clusters, and long-term engraftment (3 to 6 months) (Binder et al., 2015; Dettmann et al., 2014; Hotta et al., 2013). A shortcoming of neurosphere implantation is that the numbers of cells and the area they occupy are limited compared to what can be achieved through injection of a single-cell suspension. Implantation of 2-3 neurospheres has been described (Hotta et al., 2013), as well as microinjection of ~20 neurospheres through a 30 G needle (Dettmann et al., 2014). Since the size of neurospheres can vary considerably, and each neurosphere may contain only ~10,000 cells (Almond et al., 2007), the small numbers of cells that can be delivered is a technical limitation of this approach and substantial optimization is needed to make this a clinically viable strategy.

6.3. Serosal application

A recent study demonstrated the application of human gut-derived neural progenitor cells in a biodegradable fibrin matrix onto the serosal surface of mouse intestine that had been chemically denervated (Hetz et al., 2014). Integration of transplanted cells occurred in small ganglion structures predominantly located within the longitudinal muscle layer. The advantages of this approach are its easy performance and the potential to cover large areas using the fibrin matrix, which has been approved by FDA and is available as a spray, referred to as "fibrin glue." A potential drawback is that the formation of fibrin via the clotting cascade may block cell migration out of the matrix. The authors observed limited penetration of cells through the longitudinal muscle to reach the myenteric plexus (Hetz et al., 2014). It remains unknown if the transplanted cells located in the longitudinal muscle will be able to restore gut function. It is also unclear if injury to the bowel wall during chemical denervation impacts the likelihood that transplanted cells will migrate into and engraft in the bowel. Chemical injury may alter inflammatory mediators or the extracellular matrix as well as other cells in the bowel wall, making it difficult to know if this approach will work in uninjured tissue.

A number of bioengineering approaches have been used for delivery for cardiac, retinal and corneal cell replacements (Cutts et al., 2015; Kundu et al., 2014; Ozcelik et al., 2014). The use of a natural or synthetic biodegradable matrix, usually as a membrane seeded with stem/progenitor cells, can provide the appropriate milieu for cell growth, and when placed directly on the target or diseased site of an organ (sometimes termed a 'patch' or 'wrap'), resulting in efficient cell engraftment and homogenous cell distribution. Replacement of the missing or impaired cell types in diseased tissue through appropriate engraftment and differentiation of delivered progenitor cells may ultimately restore activity to the affected tissue. In the field of ENS cell therapy, Xu and colleagues fabricated biodegradable elastomeric fibrous mesh that supports proliferation and differentiation of enteric neural crest cells to form ganglia-like cell clusters without significant cell toxicity (Xu et al., 2013). Enteric neural crest cells grown on this synthetic membrane could be transplanted to the external muscle layer of the mouse distal colon *in vivo* and were found to colonize the appropriate gut layer 3 weeks following implantation concomitant with the degradation of the mesh. Similar to the issue described above for serosal cell application, it is unclear if cells will be able to populate the intermuscular layer when the mesh is applied *in vivo* on the intestinal serosa. However, serosal penetration was achieved when the mesh was applied to quail embryo colon in organ culture (Xu et al., 2013). The simplicity of the 'membrane wrap' technique for delivering ENS stem cells recommends it for further study as it combines ENS stem cell culture with the application step, and could represent a less invasive and effective approach to cover the large area of aganglionic gut seen in HSCR disease.

6.4. Intraperitoneal injection

Rodent p75 and α4 integrin selected neural crest-derived cells injected intraperitoneally appear to engraft into the postnatal intestine of mice with Hirschsprung-like disease (Martucciello et al., 2007; Tsai et al., 2011). This relatively non-invasive approach could be done using only local analgesia in the clinical setting. However, it seems impossible to target the area where the cells are needed. Tsai et al (2011) observed that intraperitoneally injected enteric neural crest stem cells preferentially colonized the small intestine rather than the large intestine (Tsai et al., 2011), suggesting that colonic aganglionosis is unlikely to be a candidate for this approach. Interestingly about 10% of the engrafted cells were found in gut epithelium or lamina propria with most cells in the region of the myenteric or submucosal plexus. Although transplanted cells were not observed in liver or kidney three weeks after intraperitoneal injection, further evaluation will be required to guarantee the safety of this approach, as there is the theoretical potential to form neural crest-derived tumors in other organs.

6.5. Vascular approach

Intravascular cell administration has been described extensively for cell therapy after myocardial infarction. Numerous clinical trials have already been conducted for the last three decades and a recent meta-analysis has reported intracoronary cell therapy resulted in a moderate improvement of left ventricular systolic function and a reduction in recurrent myocardial infarction at 6 months following the intervention (Delewi et al., 2014). Although there is no published study about intravascular

administration of ENS cell-based therapy via intravascular injection, the mesenteric artery could be used to deliver cells to the gut wall, in contrast to intravenous injection where transplanted cells would be delivered to all organs. Advantages of this approach include potentially homogeneous distribution of cells in the diseased segment of gut if cells are injected into the appropriate feeding artery. However, previous studies using radiolabeled cells to evaluate cell distribution following intracoronary delivery revealed only 2–6% of injected cells were retained in the heart at 24 h (Forest et al., 2010; Hofmann et al., 2005). It has been shown that molecules that guide immune cell trafficking after brain injury stimulate homing of neural stem cells into brain parenchyma after intravascular delivery (Pluchino et al., 2003). However, it is unknown if ENS stem cells can home like immune cells, and whether the necessary chemotactic factors are available in the aganglionic gut environment in people with Hirschsprung disease.

7. How do we measure cell transplantation success?

Ultimately, the "successful" transplantation of stem cells/progenitors will be defined by the ability of these cells to improve gut function. The most critical functional improvements are defined in points A1–5 below. In points B1–8 we also define a series of anatomic and neurochemical analyses that will determine the degree to which the transplanted cells integrate into the bowel and mimic the normal ENS, but restoration of function may not require restoration of normal anatomy or a normal complement of neurons. In human patients, functional studies are probably the only viable assessment of transplant success.

7.1. Animal models

(A) Functional assays and survival. The following assays provide evidence that transplanted cells have generated functional neurons, improved gut function and exhibit long-term survival:

1. *Ex vivo studies to show that graft-derived neurons are electrically active (can fire action potentials) and receive inputs.* This is important as developing enteric neurons (Hao et al., 2012) and adult neural stem cells (Moe et al., 2005) can express neuronal markers without being electrically active. These assays could be performed using a variety of electrophysiological and functional imaging approaches including sharp electrode intracellular recordings, patch clamping (Hao et al., 2012), calcium (Hao et al., 2011) or voltage-sensitive imaging.
2. *Ex vivo studies to show that graft-derived neurons functionally innervate the muscle.* Functional integration can be demonstrated by stimulation of graft-derived neurons using optogenetics or electrical field stimulation (if there are no non-graft-derived neurons in the vicinity). Stimulation of graft-derived neurons should result in (a) contraction and/or relaxation of the circular muscle as assessed by contractility studies in organ bath experiments or spatiotemporal mapping, and/or (b) the presence of excitatory junction potentials (EJPs) and inhibitory junction potentials (IJPs), the electrical events underlying contractions and relaxations respectively, in electrophysiological studies in opened preparations of recipient gut.
3. *Ex vivo studies to show that graft-derived neurons establish or contribute to circuitry for mediating complex motility patterns appropriate for the gut region.* Gastrointestinal motility patterns are region-specific. For example, mixing and emptying in the stomach, mixing and propulsion in the small intestine, and propulsion in the colon are distinct patterns of contraction and relaxation necessary for proper bowel function. Analysis of bowel motor function after transplantation could be performed using spatiotemporal mapping, calcium imaging, or simultaneous tension recordings from multiple sites.
4. *In vivo studies to show that when transplanted into animal models of human disease, appropriate function is improved.* For example, following transplantation into the colon of a HSCR animal model, propulsive gut motility (including coordinated contraction and relaxation) would occur in the previously aganglionic, tonically contracted region. Depending on the disease model, "gut motility" might be assessed by measuring total GI transit (*e.g.* carmine dye administered by gavage (Sasselli et al., 2013)), gastric emptying (*e.g.* using rhodamine B dextran), small intestine transit (*e.g.* using rhodamine B dextran), time to expel a glass bead inserted into the rectum, pellet counting, esophageal manometry, anorectal manometry, or gavage of barium contrast and then X-ray imaging of anaesthetized animals to determine rate of propulsion and to quantify contractile activity (Der-Silaphet et al., 1998). Following transplantation into a HSCR animal model, post-mortem examination could also determine whether there is a megacolon. A study of *Ednrb*$^{-/-}$ mice, a mouse model of HSCR, suggested that the intestinal microbiome plays a role in the development of Hirschsprung-associated enterocolitis (HAEC) (Pierre et al., 2014). Hence, investigations of the microbial profile of recipient animals to determine if there is a restoration of "normal" microbial profile would also be informative.
5. *Long-term survival of graft-derived cells (at least 6 months).* It would be valuable to demonstrate that transplanted cells survive in the bowel wall. While the ideal human therapy should provide benefit for decades, at least 6 months of cell survival and improved function in animal models is an achievable and valuable endpoint.

(B) Structural, neurochemical, neurogenesis and survival assays: the following assays will provide evidence that transplanted cells have generated an ENS that is similar to that generated during normal development:

1. *Migration of transplanted cells away from transplant site and colonization of normal gut locations (myenteric and submucosal regions), but not ectopic locations.* Ectopic locations to be examined should include adrenal glands, lumbar sympathetic ganglia (common primary and secondary sites of neuroblastoma, a tumour of neural crest-derived cells), other abdominal organs and lymph nodes. These experiments require that the transplanted stem/progenitors express genetic markers distinct from the recipient.
2. *Expression of pan-neuronal and glial markers by transplanted cells.* Pan-neuronal markers should include PGP9.5, HuC/D, neuron-specific enolase (NSE) and Tuj1, while glial markers should include GFAP, S100β and Sox10 (Hotta et al., 2013).
3. *Formation of, or contribution to, ganglia* (clusters of neurons and glia) by graft-derived neurons that are similar in size to endogenous ganglia in the relevant gut region.
4. *Expression of enteric neuron subtype markers, and in similar proportions to the normal ENS in that gut region.* In all mammalian species that have been examined to date, excitatory motor neurons are cholinergic (express the synthetic enzyme, choline acetyltransferase, ChAT, and the vesicular acetylcholine transporter, VAChT) and also contain tachykinins such as substance P, while inhibitory motor neurons express neuronal nitric oxide synthase (nNOS) and vasoactive intestinal peptide (VIP). Markers of intrinsic sensory neurons and some populations of enteric interneurons vary between species, but could also be examined.
5. *Absence of markers of inappropriate non-neuronal lineages* (*e.g.* markers of osteogenesis, melanocytes, cartilage, adrenal medulla, smooth muscle, adipocytes, interstitial cells of Cajal (ICC),

PDGRF-α+ fibroblasts) or inappropriate neuronal lineages (*e.g.* Brn3.0, which is expressed by neural crest-derived dorsal root ganglion neurons).

6. *Incorporation of S-phase markers such as EDU or BrdU by graft-derived neurons after transplantation* of stem/progenitor cells would show that neurons have been generated after transplantation. However, it is possible that function could be restored simply by transplanting neuronal precursors or neurons (Hotta et al., 2013).
7. *Projection of axons of graft-derived neurons to normal sites* (circular muscle, other ganglia, mucosa), and *expression of appropriate neurotransmitter* synthetic enzymes within varicosities (see point #4 above) and expression of synaptic proteins (see point #8 below).
8. *Electron microscopy, high resolution confocal microscopy or super-resolution microscopy studies to show that the axons of graft-derived neurons form synapses or close appositions with other neurons, glia, PDGFR-α+ fibroblasts, ICC and circular muscle cells.* Ultrastructural studies will require pre- or post-embedding immunolabeling to identify specifically axons of graft-derived neurons (for example, use of antisera to GFP if the transplanted cells express GFP). For non-ultrastructural studies, expression of closely apposed pre-synaptic (*e.g.* SNAP25, synaptotagmin, synaptophysin, synaptobrevin, neurexin, syntaxin) and post-synaptic (*e.g.* PSD95, neuroligin) proteins would provide support for the presence of synapses.

7.2. Human patients with enteric neuropathies

In patients into whom progenitors have been transplanted into the bowel, changes in patients' Quality of Life scores should be assessed, if possible. The following tests would indicate restoration of function:

1. *HSCR infants:* Improved passage of bowel movements without abdominal distension, bilious emesis, growth failure or enterocolitis. Studies to determine whether there is a restoration of "normal" microbial profile in the stool would also be informative.
2. *Adults (and children) with esophageal achalasia:* Improved swallowing without retained fluid or food in the esophagus. Esophagus function can be assessed using swallow tests with radio-opaque contrast and with 3D high-resolution oesophageal manometry, particularly focusing upon the gastro-oesophageal junction. Esophageal pH and impedance monitoring would also be useful to assess the frequency of gastroesophageal reflux after cell transplantation.
3. *Adults with gastroparesis caused by defective enteric neurons;* gastric emptying studies or antro-duodenal manometry are valuable ways to assess gastric function.

8. How can cell safety be assessed?

Much current work addressing the possibility of stem cell therapy for HSCR and other intestinal motility disorders has focused on the use of enteric neural progenitor cells harvested from the gut itself. Although this approach offers the possibility of autologous therapy, most current methods involve significant periods of *ex vivo* culture during which there is the potential for the appearance of genetic variants. So far, there has been little consideration of possible safety issues beyond assessing the spread of transplanted cells outside the gut and limited studies of tumor formation. With the need to perform detailed quality control for each patient, the costs for such autologous transplants are potentially high. On the other hand, human Pluripotent Stem Cells (hPSC), whether embryonic stem (ES) cells, or induced pluripotent stem (iPS) cells, present a versatile source of different cell types for transplantation in diverse regenerative medicine applications, and the possible safety issues associated with the acquisition of genetic or epigenetic changes is under active consideration (Goldring et al., 2011).

hPSC are currently being tested in a number of clinical trials in which hPSC-derived retinal pigment cells are being transplanted to treat age related macular degeneration. Other conditions for which progress is being made towards clinical trials include Parkinson's disease, Huntington's disease, type 1 diabetes and spinal cord injury. The potential of hPSC for producing enteric neurons for the treatment of Hirschsprung disease is now apparent with the development of protocols for producing neural crest cells (Menendez et al., 2011), the precursors of enteric neurons. Since hPSC can be maintained and expanded indefinitely, they offer distinct advantages over somatic stem cells in terms of the scale and reproducibility with which derivative cells can be produced. A single established hPSC line could, in principle, be used to provide cells for treating many hundreds or thousands of patients, whereas for somatic cells multiple isolates would most likely be required. On the other hand, autologous transplants would avoid immune rejection and the need for immunosuppressive therapy. The use of iPS technology is appealing in this context. More insidious, however, is the propensity of pluripotent stem cells to acquire genetic and epigenetic changes upon long-term culture and expansion (Baker et al., 2007; Draper et al., 2004; International Stem Cell et al., 2011). Such changes may reduce the efficacy of generating specific derivative cell types, or could potentially compromise safety, for example promoting tumour growth.

Although early passages of newly derived human ES cells typically exhibit a normal diploid karyotype, on prolonged passage they may acquire non-random changes to their chromosome constitution. The most common changes seen are gains of chromosome 1, 12, 17, or 20, either as extra copies of the whole chromosome, or as translocated fragments, although tandem duplications also occur (Baker et al., 2007; Draper et al., 2004; Nguyen et al., 2014; Taapken et al., 2011). It is also notable that gains of the long arm of chromosome 17 and the short arm of chromosome 12 are frequent in embryonal carcinoma cells, the malignant counterparts of ES cells and the stem cells of germ cell tumours. Human iPS cells are likewise prone to gains of the same chromosomal regions as ES cells (Taapken et al., 2011), and these observations were confirmed in a comparative study by The International Stem Cell Initiative (ISCI) of the genotypes of cells from early and late passages of 122 human ES and iPS cells, as well as from surveying literature reports (International Stem Cell et al., 2011). In addition, the ISCI study recorded examples of repetitive genomic losses affecting regions of chromosomes 10, 18 and 22. Occasional karyotypic changes affecting almost all other chromosomes have also been reported, but the changes are sporadic and form no discernible pattern, although it is notable that changes affecting chromosome 4 have almost never been reported.

The frequency of the commonly observed changes suggests that these genetic changes offer cells a selective growth advantage. This was confirmed by trials in which cultures of diploid hPSC were spiked with a small proportion of karyotypically abnormal cellsthat then took over the cultures within a very few passages (Olariu et al., 2010). In the case of chromosome 20 gains it has been possible to identify a gene, *BCL2L1*, that appears to drive the selective advantage by limiting apoptosis of hPSC during passaging (Avery et al., 2013; Nguyen et al., 2014). This gene lies in a short region of chromosome 20 that is subject to frequent amplification but often too short to be detected by standard G-banding karyotyping. It can be detected by fluorescence *in situ* hybridization (FISH) with appropriate probes, or by SNP or CGH array hybridization. Frequent screening of hPSC lines by these techniques is warranted, as the amplicon is often present in otherwise karyotypically diploid cells – in the ISCI study, the amplicon was

detected by SNP array in 22 out of 79 lines that were otherwise karyotypically diploid. These high-resolution techniques may also reveal copy number variations (CNVs), small regions of genomic gain or loss, or rearrangement elsewhere in the genome, but their significance remains unclear. Some CNVs have been identified on chromosomes 12 and 17, but repeated gains at specific loci are elusive, and some certainly reflect variants normally observed in the human population (International Stem Cell et al., 2011; Laurent et al., 2011). The failure to identify commonly affected genes, apart from *BCL2L1* on chromosome 20, does raise a question about the simple hypothesis that, in general, the selective advantage provided by specific chromosomal gain, or loss, may be attributed to the altered expression of a single gene. It may be that more complex mechanisms must be sought.

Epigenetic changes also occur in hPSC, but repetitive changes are not well documented. In the ISCI study, extensive changes in the DNA methylation of many genes were noted but no pattern emerged that was consistent across cell lines (International Stem Cell et al., 2011). Changes in the expression of some imprinted genes suggested erasure of imprinting, but it has also been reported that the imprinted genes of human ES cells are relatively stable in comparison to those of mouse ES cells (Rugg-Gunn et al., 2005). Perhaps the most widely observed epigenetic change in hPSC is the loss of X-inactivation. While the presence of two active X chromosomes may indicate of a primitive or naïve state for hPSC, many female hPSC appear to have an inactive X chromosome (International Stem Cell et al., 2007). In some cases, this inactive X seems to be re-activated (Enver et al., 2005) in a way that is permanent and non-physiological, a phenomenon that has been called erosion of inactivation (Mekhoubad et al., 2012).

Although these observations of genetic and epigenetic instability do raise safety concerns about the use of hPSC derivatives for regenerative medicine, their real significance remains unclear. Much discussion has focused on the danger of the formation of teratocarcinomas, in which undifferentiated stem cells with malignant potential persist. However, in any likely treatment it will be specific differentiated derivatives or their progenitors, not the undifferentiated stem cells that will be transplanted. So it is the potential effects that genetic changes will have on the behaviour of the differentiated cells that must be considered, (Goldring et al., 2011). On the precautionary principle, hPSC lines with overt karyotypic changes should not be transplanted to patients. However, as higher resolution screening techniques are used, genetic variants will almost inevitably be uncovered in all cell lines to the extent that the terms ‘normal’ and ‘abnormal’ lose their meaning: not only do variants that are detected in cultured hPSC occur in healthy humans (International Stem Cell et al., 2011) but normal tissues within individuals may also harbor karyotypically variant cells, although their significance is unclear (Knouse et al., 2014). Further, the ability of screening techniques to detect variant cells in a population is limited, so that cultures scored as lacking specific chromosomal variants may nevertheless harbor small undetected populations of variant cells.

The future development of hPSC for regenerative medicine will require the assessment of the effects of specific genetic changes that arise in undifferentiated hPSC on the function and behaviour of the derivative cells required for a particular application, such as for Hirschsprung disease treatment. It is also worth considering that some variants might themselves provide advantages for regenerative medicine. For example, the anti-apoptotic effects of BCL2L1 might enhance the engraftment of cells that carry the chromosome 20 amplicon. In any case, it is likely that the consequences of particular variations will vary between applications and so will require assessment on a case by case basis. Although genetic variation in hPSC is common, it is noteworthy that in the ISCI study 79 out of 120 lines studied in early and late passage retained a normal karyotype throughout and the recurrent abnormalities, whether cytogenetic or CNV, were detected in less than 50% of the cell lines. Such changes also occur in murine ES cells, but these cells have long been used successfully for the production of germ line chimeras and viable transgenic mice. Therefore there is every reason to expect that if hPSC for human clinical applications are properly monitored and the consequences of specific variants assessed, it will be possible to use these cells safely for regenerative medicine. Less is known currently about the extent to which these safety issues also apply to autologous adult stem cells, though if these require less *ex vivo* culture, the risks for adverse genetic change may be less than for long term cultured hPSC. However, that advantage is tempered by the greater difficulties of access and standardization.

Looking further towards clinical application of cell therapy for enteric neuropathies it is clear that rigorous standards, in both the generation and application of any cellular therapeutic, are required to ensure core standardization of treatment across multiple centers, at the local, national and international level. Addressing the challenges set out above will allow for development of efficient protocols for the sourcing of stem cells, their expansion, the method of transplantation and analysis of successful outcomes in patients via clinical trails. However, standardization and comparability at each step is critical, not only for safety but to enable comparison between techniques. Ultimately, it will be important that any treatment with cellular products be standardized across centers to allow for approval by the various national regulatory bodies in terms of quality, safety and efficacy. Considerable interaction between the field and these regulatory bodies will be required to determine specification and quality standards, together with providing clear evidence of consistency across processes.

9. Conclusions: the prospect of human trials

As documented across all of the sections above, the last decade has yielded significant progress in the field of enteric neural stem cells for therapy. Not only have we witnessed the development of robust and reproducible methodologies to facilitate the harvesting and propagation of therapeutic cells, their potential and safety is being tested in the context of established models of disease. More importantly, there is now a critical mass of researchers addressing the many challenges that remain and validating emerging techniques and findings. This, for the first time, has brought into view a real prospect of clinical application and ‘first in man trials’.

What would such trials look like? Although refinement is clearly needed, the harvesting of human-derived enteric neural cells for transplantation has already been established by a number of groups. Adaptation of protocols to satisfy national and international regulatory bodies and the development of approved clinical grade ‘medicines’ should not prove a major problem as a number of stem cell therapies are already established in clinical practice. Initial trials of cells designed for therapy of enteric neuropathies may be best instituted into non-diseased intestine of ‘volunteers’ with terminal diseases such as cancers not affecting the transplanted intestine, or into paediatric patients whose disease is severe and currently available interventions such as surgery offer no real prospect of improvement or are deemed to carry excessive risk. In the former, the recipient intestine is likely to be harvested at a future timepoint to facilitate assessment of cell viability, spread, functional integration and safety. In the latter, functional improvement would provide the outcome measure but ethical consideration would need to be given to the trial of such therapies in the context of increasingly safe life sustaining interventions such as parenteral nutrition and improving reported outcomes of intestinal transplantation. The gut is a huge immune organ and immunological rejection will no doubt provide an obstacle to cell transplantation.

Immunosuppression is well established clinically to induce tolerance of grafts but perhaps a more attractive solution would be the use of autologous transplantation. The validation of harvesting cells by endoscopy would favour this strategy allowing also for the delivery of therapeutic cells into diseased segments of bowel. Inherent cell dysfunction of cells harvested from affected bowel may limit this application unless transplanted cells retain sufficient function or can be delivered in large numbers or genetic rescue is feasible. Even if restoration of genetic normality is not possible some form of cellular manipulation is likely to be required to direct appropriate differentiation and engraftment of cells. Arguably the biggest challenge before contemplation of trials in man is the need for deep characterization of human enteric neuropathies. Many of these conditions remain poorly defined. Only then can one realize the precise requirements to be addressed by the transplanted therapeutic neural stem cells.

Accepting that the coming years will need to address and overcome a number of key challenges the combined efforts of a consortium bring the dream of curative therapies for enteric neuropathies closer to reality.

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