CELL THERAPY FOR GI MOTILITY DISORDERS: COMPARISON OF CELL SOURCES AND PROPOSED STEPS FOR TREATING HIRSCHSPRUNG DISEASE

Lincon A. Stamp

Department of Anatomy and Neuroscience, University of Melbourne, 3010, VIC, Australia

Running Title: CELL SOURCES AND KEY STEPS FOR CELL THERAPY FOR GI MOTILITY DISORDERS

Correspondence: lstamp@unimelb.edu.au
Abstract

Cell therapeutic approaches to treat a range of congenital and degenerative neuropathies are under intense investigation. There have been recent significant advancements in the development of cell therapy to treat disorders of the enteric nervous system, enteric neuropathies. These advances include the efficient generation of enteric neural progenitors from pluripotent stem cells and the rescue of a Hirschsprung disease model mouse following their transplantation into the bowel. Further, a recent study provides evidence of functional innervation of the bowel muscle by neurons derived from transplanted ENS-derived neural progenitors. This mini-review discusses these recent findings, compares endogenous ENS-derived progenitors and pluripotent stem cell-derived progenitors as a cell source for therapy, and proposes the key steps for cell therapy to treat Hirschsprung disease.

Introduction

The enteric nervous system (ENS) is composed of an extensive network of neurons and glia embedded within the gastrointestinal (GI) tract and it plays a vital role in nutrient and water absorption, hormone secretion and gastrointestinal motility (25). Most of the ENS is derived from vagal (caudal hindbrain) neural crest cells, which migrate into and then along the bowel during embryonic development (7). This migration occurs over a long migratory route due to the growth of the gut, and consequently occurs over extended periods of gestation; ~5 weeks (or 12.5%) of gestation in humans (68) and 5 days (or 25%) of gestation in mice (40). In addition to vagal neural crest, there are two other minor sources of enteric neurons: Sacral neural crest cells contribute some neurons to the distal bowel (10, 39), although sacral cells cannot compensate for a loss of vagal neural crest cells (8), and Schwann cell precursors, which enter the bowel via extrinsic nerves during late embryonic and postnatal development, and have recently been shown to give rise to some enteric neurons in the small and large intestines (67). The resulting ENS is a complex interconnected network containing approximately 500 million neurons in humans; more than the spinal cord (24, 26). Gut motility disorders can arise from the loss of subtypes of enteric neurons as occurs in some forms of diabetic gastroparesis, achalasia and Chagas disease (28, 42, 50, 58), or the congenital absence of the ENS, which occurs in Hirschsprung disease (6, 12, 31, 44, 49, 54). GI motility disorders caused by enteric neuropathies are some of the most clinically challenging GI conditions to manage (18-20, 42) and thus there has been considerable interest in the potential of cell therapy to treat such disorders (9, 11, 28).

During development, enteric neural crest cells colonise the gut by migrating through an undifferentiated loose mesenchyme of the gut tube (32). However, for cell therapy, transplanted progenitor cells will have to migrate in a mature, differentiated and layered muscular gut tube. Moreover, key signalling molecules that drive the migration of enteric neural crest cells during development may not be expressed at the same levels in the postnatal gut as they are in the embryonic gut (32). Despite these challenges, many studies have shown migration of progenitors following engraftment in the postnatal gut (15-17, 22, 35, 46, 62), although manipulation of signalling pathways might be required to enhance engraftment outcomes (48).

Cell therapy requires an accessible source of ENS progenitor cells to transplant into the bowel. Three recent studies have demonstrated the generation of enteric neural progenitors from human pluripotent stem cells (PSCs), utilising them for transplantation in small animal models, or for in vitro
modelling of Hirschsprung disease (22, 46, 69). Fattahi et al (2016) and Li et al (2016) showed that enteric neural precursors could be generated from human PSCs, and following transplantation into the colon of adult mice, the precursors migrated and generated enteric neurons. Moreover, transplantation of human PSC-derived enteric neuron precursors into the colon of a mouse model of Hirschsprung disease, mortality was rescued (22). Some of the aforementioned studies also utilised PSC-derived enteric neural progenitors with Hirschsprung-associated gene mutations to model the disease-in-a-dish (22, 66, 69). These recent, ground-breaking studies demonstrate the potential of pluripotent stem cell technology to treat GI motility disorders. As attempts to develop cell therapeutic approaches to treat enteric neuropathies gain momentum, it is important step back and assess the current standing and future directions of this growing field of regenerative medicine. Recent reviews of the field (9, 11) have provided comprehensive overviews of the GI motility disorders that could be targeted by cell therapy and of studies using animal models of the potential of cell therapy to treat motility disorders. This mini-review discusses recent findings using human PSCs to generate enteric neurons and compares PSC-derived cells to endogenous enteric neural progenitors as a source of enteric neurons for cell therapy. A set of key steps for the treatment of Hirschsprung disease using cell therapy is also proposed.

Comparison of endogenous ENS-derived with pluripotent stem cell-derived enteric neural progenitors

Endogenous ENS-derived neural progenitors – Numerous studies have demonstrated endogenous enteric neural stem/progenitor cells within the fetal and postnatal ENS of rodents and humans (4, 5, 36, 43, 52, 55). Therefore, much of the focus to date has been on the characterisation, isolation, expansion and transplantation of these resident enteric neural stem/progenitor cells into gut explants grown in vitro, or into the bowel of laboratory animals in vivo (3, 16, 35). Enteric neural stem/progenitor cells isolated from the rodent bowel are capable of migration, proliferation and neuronal differentiation in vitro (4, 43). Importantly, following transplantation into the bowel of postnatal recipient mice, enteric neural stem/progenitor cells proliferate, migrate and give rise to newborn neurons in vivo that are electrically active and display long term (up to 24 months) survival (16, 35). Given the possibility of tumour formation from engrafted stem cells, it is significant that there was no evidence of unusual mass formations within the bowel wall, and no indication that any of the transplanted cells had invaded or formed tumours in any other organs in recipient mice 4-24 months after transplantation (16, 35). Recent studies using ENS cells isolated from human fetal or postnatal bowel, including some patients with Hirschsprung disease, have shown engraftment, migration, differentiation and functional integration following transplantation into the mouse colon or co-culture with colonic muscle from Hirschsprung disease patients (15, 17, 59, 62).

Pluripotent stem cell-derived enteric precursors – Pluripotent stem cells (PSC) provide an exciting alternative to endogenous enteric neural stem cells to treat enteric neuropathies. Human embryonic stem (ES) cells and induced pluripotent stem (iPS) cells are capable of near limitless self-renewal and have the ability to give rise to any cell type, including enteric neurons (63, 65). Further, iPS cells can be patient-derived and can be readily genetically manipulated to correct disease-causing mutations (56, 70). Numerous studies have previously demonstrated derivation of the neural crest-like cells from pluripotent stem cells (13, 21, 34, 37, 41, 45). Engraftment or co-culture of the PSC-derived neural crest cells with explants of avian or rodent embryonic gut tissue resulted in the formation of neurons within the recipient gut (21, 34). Until recently though, protocols to obtain large numbers of
enteric neural progenitors from PSCs had not been developed. However, several recent studies have provided evidence of efficient generation of enteric neural progenitors from human pluripotent stem cells, which were utilised for drug screening, *in vitro* disease modelling and successful transplantation into Hirschsprung disease model mice (22, 46, 69).

Earlier studies from the laboratory of Lorenz Studer showed that coordinated inhibition of BMP, TGF-β, Activin, and Nodal signalling by small molecule dual-SMAD inhibition, as well as small molecule mediated Wnt activation, resulted in efficient production of neural crest-like cells from human PSCs (13, 14, 53). However, these cells were more like rostral hindbrain cranial neural crest cells, which typically do not give rise to enteric derivatives. Therefore, Fattahi et al (2016) first induced a vagal neural crest-like population they called enteric neural crest (ENC) precursors from human ES cells by further addition of retinoic acid. This resulted in expression of appropriate HOX code genes and the ability to migrate and colonise embryonic (chick) gut. Subsequent 3D spheroid culture and treatment of the ES-derived vagal neural crest cells with GDNF and ascorbic acid produced numerous neurochemical subtypes of neurons and glial cells (22).

Fattahi et al transplanted ~2-4 x10⁶ purified human PSC-derived ENC precursors, injected into the caecum of 2-3 week old immunosuppressed *Ednrbs-/-* Hirschsprung model mice. Strikingly, not only did the transplanted ENC precursor cells display extensive migration following engraftment, colonising distal portions of the bowel, but following transplantation of ENC precursors, lethality of the Hirschsprung phenotype was rescued in six recipient animals. Further, while not significant, there was a trend toward improved gastrointestinal transit times in *Ednrbs-/-* mice that received ES-derived ENC precursors, compared to controls. These data provide strong evidence for the potential of stem cell therapies to treat gut motility disorders. However, one caveat of this study is that the authors did not demonstrate the mechanism by which mortality was rescued nor whether the effect was long-term. It is anticipated that long-term restoration of gut motility would require functional innervation of the gut musculature and integration into the existing neuronal network of the normo-ganglionic regions of bowel.

In another recent study, Li et al (2016) demonstrated the efficient generation of enteric neural progenitors from hPSCs using an alternative induction protocol to Fattahi et al, involving embryoid body formation and ROCK inhibition, similar to that previously described (34, 46). Engraftment and differentiation of PSC-derived enteric neural progenitors was shown in explants of colonic smooth muscle from Hirschsprung disease patients (46). Transplantation into mouse distal colon was also performed, resulting in migration of 4-8 mm, which is more consistent with a previously published study using endogenous enteric neural progenitor (35), although the number of progenitors transplanted by Li et al (2016) was not reported and is likely to play a major role in engraftment outcomes. Very recently, Workman et al (2016) generated PSC-derived enteric neural progenitors using protocols that were similar to Li et al, except for the use of a Wnt agonist and retinoic acid, like Fattahi et al. Here, the PSC-derived enteric neural progenitors were recombined with PSC-derived mesenchyme and epithelial stem cell to form *in vitro* functional human intestinal tissue (69). Further, ENS regulation of organoid contractile activity was demonstrated.

The growing evidence of the potential of stem cell-based therapies for the treatment of gut motility disorders, using either endogenous enteric neural progenitors or pluripotent stem cell-derived precursors as a source of enteric neurons, is cause for great excitement. However, different cell
sources have advantages and disadvantages, which are summarized in Table 1 and some of these properties are discussed in more detail below.

**Table 1: Properties of different stem cells populations to treat disorders of the enteric nervous system**

<table>
<thead>
<tr>
<th>PROPERTIES OF STEM CELLS FOR ENS CELL THERAPY</th>
<th>Endogenous ENS progenitors</th>
<th>Embryonic stem (ES) cells</th>
<th>Induced pluripotent stem (iPS) cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autologous origin</td>
<td>Yes ([1, 3, 5, 15, 16, 33, 35, 36, 43, 52, 55])</td>
<td>No ([65])</td>
<td>Yes ([30, 63])</td>
</tr>
<tr>
<td>Capacity for expansion</td>
<td>Limited ([43, 47, 48])</td>
<td>Infinite ([57, 65])</td>
<td>Infinite ([63])</td>
</tr>
<tr>
<td>Demonstrated long-term survival</td>
<td>Yes (~24 months in mouse models) ([16])</td>
<td>Not yet (&lt;3 months) ([22])</td>
<td>Not yet (&lt;3 months) ([22])</td>
</tr>
<tr>
<td>Safety (no tumour formation)</td>
<td>Yes (no tumour formation 24 months after transplantation) ([16, 35])</td>
<td>Yes (no tumour formation 3 months after transplantation) ([22])</td>
<td>Not yet demonstrated</td>
</tr>
<tr>
<td>Neuronal differentiation</td>
<td>Yes; multiple neuronal subtypes ([1, 15, 16, 33, 35, 43, 52])</td>
<td>Yes; multiple neuronal subtypes ([22])</td>
<td>Yes; multiple neuronal subtypes ([22, 46, 69])</td>
</tr>
<tr>
<td>Directed differentiation of stem cells to generate specific enteric neuron subtypes</td>
<td>Not yet demonstrated</td>
<td>Not yet demonstrated</td>
<td>Not yet demonstrated</td>
</tr>
<tr>
<td>Functional neurons following transplantation into the bowel of rodents</td>
<td>Yes; action potentials, fEPSPs and calcium transients ([16, 35, 62])</td>
<td>Not yet demonstrated</td>
<td>Not yet demonstrated</td>
</tr>
<tr>
<td>Integration of transplanted cells</td>
<td>Yes ([16, 62])</td>
<td>Not yet demonstrated</td>
<td>Not yet demonstrated</td>
</tr>
<tr>
<td>Restoration of GI function in animal model of enteric neuropathy</td>
<td>Not yet demonstrated</td>
<td>Not yet demonstrated</td>
<td>Not yet demonstrated</td>
</tr>
<tr>
<td>Rescue of mortality in rodent Hirschsprung disease model following transplantation</td>
<td>Not yet demonstrated</td>
<td>Yes ([22])</td>
<td>Not yet demonstrated</td>
</tr>
</tbody>
</table>

*Potential for autologous, patient-derived stem cells* — Unlike some cell therapies, in which transient paracrine effects of transplanted cells is sufficient to induce long term changes in disease states (27), it is almost certain that the long term survival and integration of graft-derived enteric neurons will be essential for treating infants with Hirschsprung disease. Therefore, avoiding immunological rejection or ongoing use of immunosuppressants using autologous cells is likely to be beneficial. Endogenous ENS-derived neural progenitors can be isolated from the ganglionated regions and transition zone of bowel of patients with Hirschsprung disease ([51, 59]). These autologous progenitors could then be used for engraftment into the aganglionic portions of bowel in Hirschsprung disease patients. Similarly, cells isolated from Hirschsprung disease patient skin biopsy or blood sample could be reprogrammed to iPS cells and subsequently to enteric neural precursors
for autologous transplantation. However human ES cells, without using controversial somatic cell nuclear transfer techniques, could not be patient-derived.

*In vitro expansion of appropriate stem/progenitor cells for transplantation* — As discussed above, Fattahi et al (2016) injected 2-4 x10⁶ human ES-derived progenitors into the mouse caecum, and the transplanted cells colonised the entire colon. Thus, for transplantation into the human aganglionic Hirschsprung disease bowel, it is clear that extremely large numbers of cells will need to be generated to colonise the affected region and generate an appropriately dense enteric neural network. PSC populations have the distinct advantage of unlimited self-renewal (63, 65), therefore allowing the massive expansion of the cells in the pluripotent state prior to induction of the enteric neural precursor fate and subsequent transplantation. However, endogenous ENS-derived progenitors will require massive in vitro expansion in the progenitor state following their isolation. Patient-derived cells may also be defective in their proliferative or migratory ability due to the causative Hirschsprung disease gene mutations. Therefore ENS-derived progenitors will likely require in vitro manipulation either with exogenous factors known to play roles in ENS progenitor proliferation and migration (48), or by genetic manipulation.

**Directed differentiation to specific neuronal subtypes** — Cell therapy for Hirschsprung disease will require the generation of a full, functional enteric nervous system from transplanted stem/progenitor cells to restore motility to the aganglionic bowel. However, other enteric neuropathies, such as achalasia and some forms of gastroparesis, involve the loss of a specific subtype of enteric neuron (28, 42, 50). In these cases, it may be beneficial to bias the differentiation of the progenitors towards a particular enteric neuron subtype, or the precursors of that subtype, such as neuronal nitric oxide synthase (nNOS) neurons for achalasia. Methods for the efficient directed differentiation towards particular enteric neuron subtypes have not yet been developed.

**Long-term survival of grafted cells**— While enteric neural progenitors are present in the ENS, there is normally very little turnover of neurons in the gut (38). Therefore it will be important to demonstrate the long-term survival of graft-derived neurons, maintenance of their neural networks and restoration of gut function. Cooper et al have shown long-term survival of transplanted ENS-derived neural progenitors in wildtype (ganglionic) recipient mouse colon, up to 24 months (16). It will be important to also demonstrate long-term survival of ENS-derived neural progenitors in aganglionic regions of bowel in Hirschsprung disease model rodents. Human PSC-derived cells have not yet been shown to survive beyond 3 months after transplantation (22).

**Demonstration of graft-derived functional neurons and circuits** — Following transplantation into the mouse colon, neurons derived from transplanted endogenous ENS cells fire action potentials and receive synaptic inputs (35) and stimulation of endogenous nerve fibre tracks results in calcium transients in graft-derived neurons, showing integration into the existing neuronal network (16). Furthermore, ES-derived precursors were shown to rescue mortality in Hirschsprung disease-model mice, but the mechanism remains unknown and the authors suggested that the effects were unlikely to be mediated by functional integration of the cells (22). Both Fattahi et al (2016) and Workman et al (2016) demonstrated functional innervation of PSC-derived mesenchyme by PSC-derived ENS *in vitro* (22, 69). However, for the sustained restoration of gut motility, stem cell-derived neurons must form functional circuits with the gut musculature *in vivo*. Using optogenetics to selectively stimulate graft-derived cells, we have recently demonstrated functional innervation of the colonic muscle of
Box 1. Proposed Steps for Cell Therapy for infants with Hirschsprung disease

1) **Create a temporary stoma** (colostomy/ileostomy) to by-pass the obstruction.
2) **Obtain tissue from patient:**
   a) Gut tissue during enterostomy or by routine endoscopy (for endogenous ENS progenitors)
   b) Skin biopsy, blood sample or other (for iPS cells)
3) Purify and **expand ENS progenitors**.
4) **In vitro manipulation** prior to transplantation
5) **Transplant progenitors** into affected distal bowel by endoscopy.
6) **Allow time for migration and differentiation** of progenitors, and **functional innervation and restoration of motility**.

Recipient mice *in vivo* by graft-derived neurons, and showed that graft-derived excitatory and inhibitory motor neurons released the same neurotransmitters as endogenous neurons (62). An important, but very difficult to determine, issue will be to demonstrate that no inappropriate neural circuits are formed. In some cases, fetal neural stem cells transplanted into the brain of Parkinson’s disease patients have been shown to generate aberrant neural circuits resulting in dyskinesias (23, 29). The formation of appropriate circuitry will be equally important for the ENS, as the neuronal circuitry that drives propulsive motility patterns is complex and it has been shown in mice that even subtle changes of axon projection patterns can cause profound abnormalities in gastrointestinal motility (60). If aberrant neural circuits are generated in the ENS, the effects on gut motility are likely to be ineffective or even detrimental.

**Restoration of neurally-mediated complex motility patterns** – Restoration of neurally mediated complex motility patterns by transplanted progenitors is still yet to be demonstrated in animal models of enteric neuropathies. Moreover, gut motility patterns are complex and region-specific, and enteric neuropathies can affect all regions of the GIT. It will therefore be important to demonstrate that graft-derived neurons contribute to the appropriate, region-specific circuitry and restoration of motility patterns.

**Key Steps to Cell Therapy for Hirschsprung Disease**

The key steps likely to be required for the treatment of infants with Hirschsprung disease using cell therapy are shown in Box 1. While these proposed steps are focused on cell therapy for Hirschspung disease, many will be applicable to treatment of other enteric neuropathies as well. However, additional keys steps, such as the directed differentiation to specific enteric neuron subtypes (discussed above) may also be required for diseases like achalasia. The first step is an important aspect of the Hirschsprung disease cell therapy process, which will be the creation of a temporary stoma to by-pass the obstruction and prevent enterocolitis. The second step is isolation of patient-derived cells. The source of these cells will depend on the stem/progenitor cell to be used for transplantation, with endogenous ENS progenitors isolated from the normo-ganglionated regions of bowel by routine endoscopy (52) or from the ganglionated margin of resected bowel during stoma creation (59). Alternatively, fibroblasts could be isolated from patient skin biopsies or other cell types from relatively non-invasive procedures and reprogrammed to iPS cells if PSC-based therapy is to be used.

Third, appropriate progenitors need to be purified and expanded dramatically. PSCs have the distinct advantage of near limitless self-renewal in the pluripotent state, meaning the expansion phase can...
be done as PSCs followed by induction to enteric neural progenitors. However, endogenous ENS progenitors will require substantial in vitro expansion in the progenitor state, and culture conditions to enable this are yet to be established. Therefore, endogenous ENS progenitors will likely require in vitro manipulation with either exogenous factors known to drive enteric neural crest proliferation during development (48) and/or genetic manipulation to improve their proliferation and engraftment outcomes. Further, depending on the disease-causing mutation and its impact on ENS function, in vitro manipulation to correct causative mutations may also be required for both endogenous and PSC-derived enteric neural progenitors. For example, mutations in RET, which encodes a receptor tyrosine kinase, are the major cause of Hirschsprung disease, with ~50% of patients having a mutation in the coding region of RET (2). During development, RET is known to play important roles in enteric neural crest survival, proliferation, migration and differentiation (31, 44, 61, 64). Therefore to generate a new, normal ENS in Hirschsprung disease patients where one is congenitally absent (the aganglionic region), RET mutations may need to be corrected using CRISPR-Cas9 technology. This technology has been used to correct disease-causing mutations in patient-derived cells for several diseases (56, 70), however RET mutations in ENS cells have not yet been corrected using this approach, and the safety of this technology for clinical use is still uncertain.

A major question that has yet to be thoroughly investigated is the optimal route for delivery of the progenitor cells to the affected regions of bowel. Most rodent studies to date have transplanted the progenitor cells as neurospheres (16, 35, 46). However Fattahi et al (2016), despite transiently growing the PSC-derived enteric progenitors as spheroids, injected 2-4 x 10^6 cells as a single cell suspension. Further, these cells were injected into the caecum, rather than the aganglionic distal colon, in studies using Hirschsprung disease-model mice. The optimal method for delivering the progenitors to patient bowel should be minimally invasive but accurate and repeatable.

Finally, time will play an important role, which can be made available to Hirschsprung disease patients following the creation of a temporary enterostomy (proposed in step 1). In addition to allowing time for the expansion of progenitors, it will also take time for the migration, differentiation and maturation of neurons and glia following transplantation of cells.

This proposed cell therapeutic approach may ultimately prove to be unrealistic in some patients, particularly those with long-segment Hirschsprung disease. An alternative approach could be to use cell therapy as an adjunct treatment to surgical resection, implanting progenitors to in the distal colon (including any residual transition zone or aganglionic muscular sleeve), to improve post-operative outcomes.

Conclusions

Significant advances have been made by numerous research groups in the development of approaches to apply neural stem cell-based therapy to treat gut motility disorders (9). The recent production of pluripotent stem cell-derived enteric neural crest precursors (22, 46, 69) and their successful transplantation into recipient mouse colon (22, 46), and our demonstration of functional innervation of the colonic muscle of recipient mice in vivo by graft-derived neurons (62), has resulted in increased awareness of the field and a consolidation of efforts to move this research toward the clinic. This should occur only after addressing some of the above outstanding questions
in animal models of enteric neuropathies and only in a coordinated effort with expert clinicians and surgeons.

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