INTRODUCTION

Enteric neuropathies exist as a wide range of human disorders, impacting variably on the gastrointestinal (GI) tract, including a number of severe motility disorders such as achalasia, gastroparesis, and slow transit constipation. Such conditions can arise from disruption of the development of the enteric nervous system (ENS) or through acquired processes, which lead to neuronal loss or the disturbance of specific neuronal signaling. Hirschsprung disease (HSCR) is one of the best characterized congenital enteric neuropathies, resulting from absence of ENS formation in a variable region of the colon, which reflects a failure of the original ENS precursors to complete rostro-caudal colonization of the developing GI tract. The absence of the ENS in HSCR patients results in constriction of the terminal aganglionic intestine from unopposed tonic muscle contraction, causing blockage and associated distention of the proximal intestine. Current therapeutic intervention, in such cases, is limited to surgical resection of the aganglionic region. Unfortunately, in a significant proportion of HSCR patients such interventions have a significant morbidity and overall poor long-term prognosis, with patients often requiring further surgical management through early childhood and adolescence. Beyond HSCR, the failure of currently available surgical techniques to provide a curative treatment across a variety of motility disorders underlines the need for alternative approaches to treat these devastating diseases.

This need, coupled with advances in our understanding of the underlying genetic and cellular elements involved in the development of the ENS have provided the impetus to investigate potential cell-based therapies as a means of replacing neurons which have...
been lost via disease processes or are absent through failures in development, in a range of GI disease models.

### 2 | IDENTIFICATION AND ISOLATION OF ENTERIC NEURAL STEM CELLS

The ENS is derived principally from a population of vagal neural crest-derived cells, which enter the foregut in humans at approximately embryonic week 4 (mice at approx. E9.5)\(^{12,13}\) and migrate in a rostro-caudal fashion to colonize the entire gut by approximately gestational week 7 in humans\(^{33}\) or E13.5 in mice.\(^{34}\) A smaller contribution to the ENS is attributed to a sacral neural crest cell (NCC) population, in mouse and avian models (and presumably in humans), which appear to enter the terminal colon and migrate to colonize this terminal region in an anal-to-oral fashion.\(^{15,16}\)

In order to fully colonize the expanding gut during embryonic development, vagal and sacral NCCs are required to both proliferate and migrate extensively. Elegant imaging studies have allowed for visualization of these processes\(^{17,18}\) demonstrating the migration of NCCs in chains with highly dynamic migratory patterns, including “trans-mesenteric” migration of enteric NCCs in the colonization of the mouse colon.\(^{19}\) This migratory pattern is highly dependent on several signaling pathways, which have been reviewed in detail elsewhere\(^{20-23}\) and as such are outside of the scope of this current review.

Of significant interest is the ability of the migratory enteric neural crest to colonize the expanding gut in terms of cell numbers. In order to enable full colonization, it is clear that a significant proliferative capacity is required to expand the relatively small population of pioneer neural crest cells entering the foregut to ultimately lead to the formation of the largest branch of the peripheral nervous system incorporating 200-600 million enteric neurons and glia.\(^{24}\) This process is tightly coordinated by Ret/GDNF signaling\(^{25}\) with the enteric NCCs undergoing significant expansion along the length of the developing gut.\(^{26}\) Additionally, early studies by Bondurand et al. highlighted the critical role of SOX10 and endothelin 3 signaling in the maintenance of multilineage ENS progenitors.\(^{27}\) The proliferative capacity and multipotent nature of these neural crest derivatives has led to the investigation of enteric neural stem cells (ENSC) as a potential tool to treat enteric neuropathies. Interestingly, ENSCs have been identified in both fetal and postnatal tissues\(^{28-30}\) including their identification in human postnatal gut samples,\(^{31,32}\) which suggested the persistence of enteric “stem-like” cells after enteric neural crest migration has ended. Critically, in both rat and mouse gut, p75\(^+\) or RET\(^+\) progenitor cells have been shown to display clonal characteristics.\(^{35,36}\) Additionally, after single cell transplantation into an organotypic gut culture system, transplanted RET\(^+\) cells were shown to give rise to large numbers of progeny capable of generating both cell lineages of the ENS.\(^{36}\) Similar experiments in an embryonic organotypic rat gut culture system suggested the expression of the homeomain transcription factor Phox2b as an alternative or additional marker of multipotent enteric NCCs. These experiments crucially demonstrated the presence of early postmigratory ENSCs within gut tissues and suggested the existence of “adult” enteric neural stem cells within mature gut, after birth.

In order to confirm the possible persistence of an ENSC pool after birth Bondurand et al. utilized a retrovirus-mediated gene transfer approach to selectively enrich and label proliferative ENSC from both embryonic (E11.5) and postnatal mouse tissue. Interestingly, both embryonic and postnatal ENS progenitor cells led to clonal colonies at a rate of approximately 25%. However, postnatally derived ENSC were found to produce smaller colonies, suggesting a temporal reduction in proliferative capacity from embryogenesis to postnatal maturity, similar to the findings of p75\(^+\) multipotent progenitors in rat gut tissue.\(^{35}\) Characterization of ENSC in this context demonstrated that clonogenic ENSCs derived from both embryonic and postnatal gut express SOX10. With increasing time in culture, these SOX10\(^+\) ENSC display expression of markers for differentiated enteric lineages including TuJ1\(^+\) neurons and GFAP\(^+\) glial cells. Hence, similar to in vivo enteric progenitors, isolated ENSC represent multipotent progenitors, which can self-renew before acquiring neurogenic or gliogenic markers prior to terminal differentiation.\(^{28}\)

While each of these preliminary studies significantly added to the understanding of both the presence and behavior of ENSC within rodents, the potential translation of these studies and identification of ENSC within human samples was postulated to represent a clinically relevant source of neuronal precursors which could be utilized for cell replacement therapy. Similar to rodent gut, fetal and postnatal human gut have been shown to contain ENSC\(^{32,37}\) with their identification within samples taken from
patients aged up to 84 years old suggesting that multipotent ENS progenitor cells are maintained throughout adult life and therefore could be manipulated to meet therapeutic needs. Interestingly, and potentially crucial in the translation of any future therapy was the finding that ENSC could be harvested from routine mucosal biopsies. This potentially raises the possibility of accessible (and repetitive) harvesting of autologous ENSC, which could be expanded in vitro prior to back-transplantation. Of note, whereas previous rodent studies utilized FACS isolation of ENSC based on p75 or RET positivity, these initial human studies relied heavily on specific culture conditions for the enrichment of ENSC. Despite these studies demonstrating the presence of ENSC within human colon, subsequent studies have suggested that isolation methods based solely on culture conditions invariably include non-NCCs, which brings into question this strategy as the first step of a potential therapeutic option. Rather, this study found that in order to isolate bona fide neurospheres, comprised exclusively of neural crest-derived cells, antibody selection with p75+ or an alternative marker is crucial.

3 | TRANSLANTATION OF ENTERIC NEURAL STEM CELLS TO GANGLIONIC COLON

Although the archetypal disease model for neural replacement therapies is Hirschsprung disease, where distal aganglionosis persists, a number of proof of principle studies have established the potential for in vivo transplantation and development of ENSC-derived neurons in ganglionated colonic segments within a wild-type murine context. Such investigations have typically utilized transgenic reporter models, under the control of enteric specific promoters to allow for the selective labeling and isolation of ENSC prior to culture.

Hotta et al. initially demonstrated isolation and expansion of ENSC from both the EdnrbLck and Ret+Gm mouse models in which all neural crest derivatives express the photoinducible protein Kikume or EGFP, respectively. In vivo transplantation of both embryonic and postnatal ENSC from these sources leads to the engraftment of donor-derived cells within the colonic muscularis. Critically, transplanted ENSC, in this context, have been shown to demonstrate greater efficiency above CNS stem cells in the generation of enteric neurons within transplanted intestinal tissues and have been shown to differentiate to appropriate enteric phenotypes including neuronal and glial lineages. Interestingly, postnatally derived ENSC were found to occupy a reduced area 4 weeks after transplantation when compared to embryonic ENSC, which may again reflect a temporal reduction in capacity with increasing developmental age. A more recent study has suggested that it may be possible to exogenously enhance the behavior of ENSC with application of various enteric growth factors. Indeed, exposure of ENSC to GDNF significantly increased neurosphere size, the distances over which neurosphere-derived cells migrate within an embryonic gut co-culture system or after in vivo transplantation, and neurogenic potential suggesting it may be possible to pharmaceutically enhance ENSC properties pretransplantation.

A key requirement of any future successful cell therapy is the development and integration of transplanted enteric neurons, which could potentially mediate motor control of intestinal segments. Initial investigations to establish the functional integration of transplanted ENSC demonstrated electrical activity within individual ENSC-derived neurons from either embryonic or postnatally derived sources via intracellular recordings. Subsequent studies have additionally demonstrated functional integration of transplanted ENSC-derived neurons using a number of approaches. Cooper et al. exclusively using postnatally derived ENSC from the Wnt1Cre/+;R26RFP/YFP mouse model as donors (Figure 1), where all NCCs and their derivatives express yellow fluorescent protein (YFP), similarly demonstrated successful integration and long-term survival of ENSC-derived neurons within wild-type ganglionated colon, up to 24 months posttransplantation. Using a calcium imaging approach, Cooper et al. demonstrated widespread functional integration of multiple cells within a transplanted neural network, suggesting an integrated circuitry between the endogenous ENS and transplanted ENSC-derived neurons develops posttransplantation. Crucially, these preliminary studies additionally demonstrate that donor-derived ENSC not only form functional neurons in vivo but also adopt the appropriate localization and that neuronal subtype specific differentiation gives rise to a repertoire of enteric neurons including ChAT, VAChT, nNOS, Calretinin, Calbindin and VIP-expressing neurons. Of interest, postnatally derived ENSC have been shown to predominantly lead to the formation of nNOS expressing neurons whereas embryonically derived

![Characterization of Wnt1Cre/+;R26RFP/YFP-derived neurospheres. Representative confocal image showing immunohistochemistry of Wnt1Cre/+;R26RFP/YFP-derived neurospheres demonstrating inclusion of neurons (TuJ1) within labeled neurospheres grown in culture after YFP FACS isolation. Scale bars represent 50 μm](Image 308x124 to 546x342)
ENSC have been shown to display similar excitatory and inhibitory neuronal lineage characteristics after transplantation. However, the mechanism for the apparent predisposition of postnatal ENSC to form nitricergic neurons is unclear at present.

Extending this characterization, a recent study utilizing donor ENSC under the control of an optogenetic reporter (Wnt1::Cre;ChR2EYFP) successfully demonstrated that ENSC-derived neurons from both embryonic and postnatal donors again form appropriate neuronal lineages including ChAT and nNOS expressing neurons, reflecting the 2 main branches of enteric neurotransmission. Indeed, posttransplantation optogenetic stimulation of donor cells in recipient colonic tissues resulted in excitatory and inhibitory junction potentials in colonic muscle cells further demonstrating donor ENSC-derived neurons can mediate motor control. Furthermore, this study demonstrates that transplanted ENSC have the ability to generate the necessary circuitry for motor control with the development of both interneurons and motor neurons in vivo. Strikingly, there again appears to be disparity in the ability of embryonic and postnatal ENSC to form interneurons, with embryonically derived ENSC giving rise to both cholinergic and purinergic interneurons, whereas postnatal ENSC generated purinergic neurons predominantly. The reasoning behind these differences is unclear at present, but such findings may have significant implications in the establishment of translational studies, whereby autologous transplantation of ENSC to form nitrergic neurons, whereas postnatal ENSC generated purinergic neurons predominantly. The reasoning behind these differences is unclear at present, but such findings may have significant implications in the establishment of translational studies, whereby autologous transplantation of ENSC harvested from postnatal gut, prior to expansion in culture and back-transplantation would be the presumptive gold standard therapeutic protocol.

While these preclinical murine transplantation models successfully demonstrate the potential for in vivo transplantation of ENSC and functional development of donor neurons, studies were further required to establish if human-derived ENSC could display similar characteristics after in vivo transplantation. Using fetal human ENSC isolated using p75 antibody selection and interspecies transplantation into an immunodeficient (Rag2−/γc−/C57) mouse model, Cooper et al. clearly demonstrated the development of human ENSC-derived enteric cell types including neurons and glia. Again similar to earlier mouse transplants ENSC-derived cells could be observed within appropriate locations forming both ganglia-like structures and projecting fibers to make connections with the endogenous ENS. Moreover, this study demonstrated the functional integration of transplanted human ENSC-derived neurons with the endogenous mouse ENS suggesting that the hypothesis of a potential cellular therapy for enteric neuropathies "holds water." However, a critical step in proving the potential for therapeutic improvement of gut motility disorders relies on the demonstration of functional rescue of a gut pathophysiology.

4 | TRANSLATION OF ENTERIC NEURAL STEM CELLS TO DISEASE MODELS

In order to test the ability of ENSC to rescue a disease phenotype, multiple groups have used a range of recipient tissues including aneural gut segments, chemical ablation approaches and genetically altered animal models of aganglionosis or neuropathology. Initial experiments carried out nearly a decade ago, using what we now understand to be sub-optimal isolation conditions for ENSC established that human gut-derived ENSCs could engrat within aneural chick gut segments cultured using chick chorioallantoic membrane (CAM) or aganglionic gut segments grown in organotypic culture. Subsequent pilot studies using this human ENSC isolation protocol coupled with in vivo transplantation to chemically ablated mouse gut tissue suggested successful integration of PGP9.5− neurons and GFAP+ glial cells indicating the presence and survival of donor cells in vivo. However, such cells did not localize within ganglia-like structures but rather appeared in isolation located in the smooth muscle layers suggesting failure of plexus regeneration after transplantation. Interestingly, this study also suggests that transplantation may have physiological effects on gut contractility. A major caveat with chemical ablation studies using benzalkonium chloride (BAC) treatment is the significant limitation in clarity about the ability to fully ablate the entire endogenous ENS in any given gut segment. Additionally, questions remain as to the possible invasion of neural fibers from the adjacent endogenous ENS, which may obscure the functional recovery provided by transplanted ENSC alone.

More recently Rollo et al. using a selective p75 enrichment approach have successfully demonstrated the isolation of human ENSC from ganglionated proximal sections of resected human HSCR colon demonstrating the potential for autologous sourcing of cells from "normal" sections of patient gut in a diseased setting. Interestingly, this study found that direct sorting from freshly dissociated colonic segments was not an effective means of propagating cells. Rather cells were cultured for up to 1 week prior to isolation. However, this culture period was not found to lead to changes in neuronal crest gene expression, suggesting that short-term culture of dissociated patient samples may provide a means to overcome some practical hurdles related to human ENSC expansion. Moreover, this study demonstrated that such cells could integrate and form neurons in autologous aneuronal gut explants obtained from the same patient critically demonstrating autologous human ENSC cell replacement is possible in "diseased" gut.

Additional studies have sought to use genetic models of aganglionosis to better recapitulate the neuropathological phenotypes seen in enteric neuropathies. Initial studies using the EDNRB−/− rat model, which displays Hirschsprung-like aganglionosis, show that after intraperitoneal transplantation, p75− selected donor ENSC from embryonic rat intestine endogenously labeled with human placental alkaline phosphatase under the transcriptional control of the Rosa26 promoter (R26-hPAP) migrate to the gut, localizing to the proximity of the submucosal and myenteric ganglia and appropriately differentiate to neurons and glial cell lineages. Similarly, unsorted murine ENSC isolated from postnatal Actb-DSRed intestine, which express the red fluorescent protein DSRed under the control of the chicken beta actin promoter, or sorted Wnt1cre/+;R26R(Fyp)YFP ENSC transplanted to the aganglionic colon of Ednrb−/− mice, either in ex vivo cultures or via endoscopic microinjection, led to
colonization and engraftment of both neurons and glia. Interestingly, endoscopically applied ENSC were observed solely within the submucosal plexus at 1 week providing further evidence that donor ENSC survive within aganglionic gut segments.\textsuperscript{48}

Hotta et al. have additionally shown that ENSC can be isolated from ganglionic segments of Ednrb\textsuperscript{tm1Ywa} intestine akin to the ideal treatment option for HSCR patients. After cell culture, lentiviral labeling and transplantation to the distal colon, these cells were found to retain their capacity for self renewal and could be observed proliferating and differentiating into neurons within the aganglionic colon after 2 weeks in vivo. Indeed, the same research group has recently demonstrated that unsorted human ENSC harvested from postnatal ganglionic HSCR bowel resections can colonize both aneural chick gut and Ednrb\textsuperscript{−/−} aganglionic colon both in ex vivo organotypic cultures and in vivo.\textsuperscript{49}

Human ENSC were observed within the gut wall, close to the site of transplantation and gave rise to both neurons and glia. These studies taken together provide further evidence for potential autologous treatment of human neuropathologies. Unfortunately, due to severe phenotypes and poor survival of aganglionic mouse lines, studies to ascertain functional rescue remain a rate-limiting factor.

In order to circumvent this issue, recent work from our group sought to utilize the neuronal nitric oxide knockout (nNOS\textsuperscript{−/−}) mouse model, which has previously been shown to display complete loss of nNOS neurons in the colon resulting in slow colonic transit.\textsuperscript{50} Transplantation of donor ENSC, derived from postnatal Wnt\textsuperscript{1cre/+};R26RYFP/YFP mice, to the nNOS\textsuperscript{−/−} colon leads to formation of YFP\textsuperscript{+} networks of transplanted cells within the distal colon (Figure 2). Despite previous transplantation studies, using equivalent ENSC sources, having reported quite modest engraftment of transplanted cells, typically colonizing approximately 5 mm\textsuperscript{2}, this study using confocal microscopy of the entire colon, demonstrated extensive trans-colonic engraftment of YFP\textsuperscript{+} donor cells up to 42 mm from the presumptive transplant site. The significant differences in colonization most likely reflect this differing technical approach rather than any changes in cellular or tissue characteristics. Hence, it is likely that future studies will need to assess colonization of target organs in a more detailed fashion at the microscopic level, rather than relying solely on stereoscopic live imaging techniques.

Significantly, transplantation of ENSC to nNOS\textsuperscript{−/−} distal bowel also led to the development of nNOS\textsuperscript{+} neurons and the restoration of nitrergic responses. Intriguingly, such transplantation also led to unexpected increases in interstitial cells of Cajal (ICC) numbers that are reduced in the nNOS\textsuperscript{−/−} colon, pointing to possible non-cell-autonomous effects of ENSC transplantation which had not previously been considered in the field. Moreover, these combined effects of ENSC transplantation led to the rescue of impaired colonic motility providing the first direct evidence that in vivo ENSC transplantation can restore function, at the organ level, in a pathophysiological disease model.\textsuperscript{51}

5 | ALTERNATIVE CELLULAR SOURCES FOR THE TREATMENT OF ENTERIC NEUROPATHIES

Together with preliminary studies of ENSC demonstrating the potential of an autologous stem cell therapy to rescue pathophysiological gut deficits, alternative pluripotent cellular options have been similarly demonstrated to have therapeutic potential. A study by Zhou and Besner demonstrated the potential deri

\begin{figure}[h]
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\caption{Colonization and integration of transplanted ENSC within the colon. Representative stereoscopic image demonstrating that after in vivo transplantation, YFP\textsuperscript{+} transplanted cells can be identified within the nNOS\textsuperscript{−/−} distal colon. YFP\textsuperscript{+} neurospheres were transplanted to the distal colon approximately 1 cm from the anal margin (red arrow) between P14-17. 4 weeks after transplantation YFP\textsuperscript{+} (green) cells are typically observed forming large anastomosing networks extending along both the longitudinal and circumferential axes in all orientations. Scale bar represents 500 \textmu m.}
\end{figure}
human ES and iPSCs. Interestingly, thorough molecular character-
ization of the cells demonstrates the ability to drive pluripotent stem cells toward an ENC fate and subsequently to terminal neural subtypes including nNOS, 5-HT, and ChAT neurons in vitro. This study also highlights the potential of this approach as a drug discovery platform. Moreover, this study demonstrates the poten-
tial rescue of a Hirschsprung phenotype with a remarkable 100%
survival of 

Ednrb−/− (SSL/LEJ) mice after transplantation of up to 4 million ENC cells to colon. Unfortunately, this study fails to provide a mechanism which may allow for this graft-mediated rescue. Hence, questions remain as to the level of aganglionosis, which is recovered in this strain of mice, and how transplantation of iPSC-derived ENC affects host colonic motility. Li et al. similarly demonstrate derivation of neural crest stem cells (NCSC) from human iPSC using an alternative approach based around FACS sorting of HNK+, and p75+, iPSC-derived NCCs. Using a gut explant co-culture system to study enteric neural differentation, this study complements that of Fattahi et al. demonstrating development of a host of enteric neural markers including VIP, ChAT, calretinin, tyrosine hydroxylase, and nNOS as well as the functional development of electrophysiologically mature neural phenotypes. This study also showed the ability of these human iPSC-derived NCSC to colonize aneural mouse and chick gut. Furthermore, engraftment of iPSC-derived NCSC within BAC-treated ganglionic and aganglionic gut tissue from human HSCR patients led to the development of enteric-like neurons and reductions in contractile frequency in ex vivo cultures. However, it is unclear how many iPSC-derived NCSC were transplanted in these conditions making any comparison difficult. One significant limitation highlighted in this study was the failure of iPSC-derived NCSC to adopt an enteric neural phenotype in the absence of gut explants despite peripheral neural differentiation culture conditions. This caveat suggests that key microenvironmental factors may provide molecular cues to allow for “correct” directed differentiation of iPSC-derived cells, which may play an important role in translation of therapy where, at present, patient-specific understanding of the intestinal microenvironment is unfeasible.

More recently, efforts have focused on tissue engineering approaches including the development of human intestinal organoids (HIOs) and subsequent implantation of pluripotent-derived enteric NCCs (ENCC). Here, using 2 differing derivation approaches, the incorporation of ES or iPSC-derived enteric NCCs within HIOs led to the functional incorporation of neuronal and glial cell types within organoid units which have previously been shown to contain mesenchymal cell types but lack a neural crest component. In both of these studies, incorporation of ENCC led to the functional development of albeit rudimentary activity, such as ENS-mediated contraction of HIOs and calcium activity within transplanted ENCC derived from a human iPSC line expressing GCaMP4. These studies critically provide evidence that is it possible to form functional intestinal units in vitro, which may play a significant role in drug discovery and patient specific preclinical testing of cellular and compound based strategies. Interestingly, Schlieve et al. additionally show that incorporation of pluripotent-derived ENCC led to significant alteration of numerous gastrointestinal tissue-specific genes. This transcriptomic alteration after supplementation of ENCC within this alternative system further supports our findings that transplanted ENCC can lead to non-cell-autonomous changes in the cellular microenvironment.

One of the critical limitations of any cellular therapeutic treatment will be the requirement to address the underlying cause of disease. In circumstances of disease, a mitigating factor in possible treatment options will be the inherent preservation of the disease causing mutations in autologously sourced cells. However, advances in gene therapy, such as CRISPR/Cas9 technology, may provide an elegant mechanism to correct disease-causing mutations providing a pool of “normal” gene-corrected ENCC or iPSC. Indeed, a recent study by Lai et al. elegantly demonstrated correction of HSCR-associated mutations in human iPSC using CRISPR/Cas9. Here, iPSC derived from a HSCR patient, displaying RET mutation, were corrected using CRISPR/Cas9. Subsequently ENCC characteristics, including neuronal differentiation capability in the presence of GDNF, were restored after a stepwise differentiation protocol. Importantly, this study highlights the capacity to correct genetic deficiencies in potential donor cells using rapidly evolving gene technologies. Such an approach could theoretically be utilized to ameliorate any defective genetic pathways (RET, EDNRB, nNOS etc.) known to cause enteric neuropathy. While this exciting development opens up novel treatment avenues it will be vitally important to fully understand the basis of disease, any potential compensatory mechanisms which may occur and the potential for off-target genetic effects before clinical application of such technologies.

6 | FUTURE CHALLENGES FOR ENTERIC NEURAL STEM CELL THERAPEUTICS

Many of the challenges which will be required for translational therapy of ENSC for use in the clinic, including harmonization of protocols and approaches across multiple research groups and centers, have been outlined in a major white paper from leaders in the field. Hence, this short review will focus on a number of pertinent new challenges, which have arisen given recent data.

Preliminary studies of murine ENSC transplantation reported modest engraftment and coverage of transplanted cells within host colon tissues. These reports, including from our laboratory, typically utilized stereoscopic live imaging techniques to analyze transplanted cell coverage of approximately 4-11 mm². Such modest coverage has dogged the field, for several years, with warranted skepticism of the potential to upscale cell treatment in order to facilitate engraftment in large regions of gut. However, our recent work has shown that this stereoscopic approach may be flawed. While this approach allows for initial visualization of large patches of transplanted cells on the serosal surface, it appears unable to adequately resolve (i) transplanted cells which have migrated away from the presumptive site of transplantation and appear in isolation/minimal numbers, (ii)
cells which have migrated into and along the muscularis or (iii) relatively dim transplanted cell processes. Using this stereoscopic approach in our most recent study, we similarly revealed approximate transplanted cell coverage of 5.2 mm². However, when whole colonic sections were subsequently immunolabeled with anti-GFP antibodies, boosting the signal from transplanted ENSC-derived cells, we revealed extensive trans-colonic engraftment via confocal microscopy.51 Hence, despite using an identical transplantation technique and identical donor ENSC, previous estimations of cell coverage may be significantly underrepresentative of actual engraftment. This finding raises interesting questions regarding the migration of ENSC in vivo. With previous transplantation reports of cell coverage, it was assumed that cells migrated from the transplantation site with enteric neural crest characteristics, ie, in migratory chains. Our finding of transplanted ENSC-derived cells in the far proximal colon raises questions as to the migratory behavior of these cells. Figure 3 highlights two possible mechanisms whereby cell migration away from the transplant site could lead to integration of ENSC-derived cells at distant sites. The current dogma suggests that transplanted cells would initially migrate into and along the gut using cues from the endogenous ENS to guide transplanted cells to their eventual site of engraftment (Figure 3, upper panel). Alternative studies have shown that there appears to be a close apposition of transplanted cell fibers with the endogenous ENS,42 supporting this theory. In this scenario, the endogenous ENS is assumed to provide a “roadmap” and molecular cues, which transplanted ENSC could follow. We speculate that a possible alternative is that a proportion of transplanted cells may use the enteric vasculature for vessel-supported migration or potentially intravasate to the enteric vasculature and re-enter the musculature at sites along the colon where they can then respond to cues from the endogenous neural network as above (Figure 3 lower panel). Previous reports have highlighted that neural crest derivatives, including neural stem cells, migrate along the vasculature in the CNS.61,62 Additionally, it has been shown that during development, migrating ENCC cross trans-mesenteric vasculature to colonize the colon.19 However, our current understanding of the molecular cues involved in this process is limited. A potential caveat to dispute these hypotheses is that safety data post-ENSC transplantation suggests that donor cells are only found within the target colonic tissue42 rather than in peripheral organs including the mesentery, which might be expected if a vascular migration phenomenon did exist. In order to progress toward possible clinical application of ENSC it will therefore be necessary to further investigate the exact migration pathways of donor ENSC in order to circumvent off-target engraftment.

More pertinent are the possible non-cell-autonomous effects, which transplanted ENSC may exert on the host microenvironment. Our recent work demonstrated that in vivo transplantation of ENSC into an nNOS-deficient microenvironment led to the development of nNOS⁺ neurons. Unexpectedly, we also observed significant increases in ICC numbers throughout the colon of these mice.51 results which appear consistent with observations of transcriptomic alteration after ENS incorporation within human intestinal organoid units.57 It is now clear that a much more detailed understanding of the effects that transplanted ENSC, be that gut or pluripotent-derived, have on the host neuromusculature is required. It is also apparent that our current understanding and phenotyping of gut motility disorders in terms of cellular or functional pathology is lacking, and future studies will be required to redress these issues in order to proceed toward clinical translation.

A final and critical challenge for the field will be to replicate the promising findings published regarding murine ENSC transplantation in the human context. Techniques used in murine studies have been directly translated to human samples with varying results. Although study of fetal human samples has progressed to show functional integration of human cells after in vivo transplantation to mouse models29 transplantation studies using postnatal human ENSC appear to have stalled, likely due to issues with their expansion after antibody selection. Proof of concept studies do indeed show the promise for isolation and expansion of human ENSC from postnatal human patient samples, however, the nonselective techniques used in these studies together with our current knowledge of the heterogeneous composition of unsorted neurospheres38 will likely prevent any translation of these protocols in their current form, without significant refinement. Realistically, for any potential treatment, it will be critical to demonstrate adequate enrichment of ENSC through antibody labeling followed by robust expansion in culture. For this to occur, alternative protocols may be required which diverge from previously published murine work and are more specifically targeted toward human stem cell culture. This could potentially require cross-disciplinary collaboration and transparent protocol exchange, within the field, to establish best practices in order to drive postnatal human ENSC transplantation to the forefront.

**FIGURE 3** Possible ENSC migration pathways in the colon after transplantation. Representative schema showing possible migration pathways that transplanted ENSC may utilize to colonize the colon. Top panel: Transplanted cells initially migrate into and along the gut using cues from the endogenous ENS to guide them to their eventual site of engraftment. Lower panel: A proportion of transplanted cells may migrate via the vascular tree supported by blood vessel molecular cues or may potentially invade the enteric vasculature and subsequently re-enter the colonic musculature at sites along the colon.

**Oral**

**Anal**
CONCLUSIONS

The past decades have seen a significant increase in our ability to isolate and manipulate stem cells for the treatment of enteric neuropa-thies. Our current understanding of ENSC offers promise of a potential autologous cell source, which may benefit a number of disease areas. However, in order to realize this potential, future studies are required to ascertain transplanted cell dynamics and the effects of transplantation on the host microenvironment. These studies, in addition to improved and more efficient protocols for the isolation of postnatal human ENSC, are fundamental priorities before this exciting cellular therapy can be translated to first-in-man studies.

CONFLICT OF INTEREST

The authors have no competing interests.

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