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RESEARCH LETTERS

Unlocking the Neurogenic Potential of Enteric Glial Cells for Hirschsprung Disease Therapy

Enteric glial cells (EGCs) form an integral part of the enteric nervous system (ENS), exhibiting a wide array of functions,[1] including control of gut motility and communication with the immune system, and play a key role as neural stem cells in the adult gut.[2] When the gut is damaged by inflammation or disease, EGCs have been found to restore the ENS, transdifferentiating into enteric neurons in vivo.[3,4] Although neurogenesis is limited under normal conditions in adults,[5,6] the neurogenic potential of EGCs holds promise for cell therapy to treat enteric neuropathies, such as Hirschsprung disease (HSCR).[7–9] HSCR, also known as congenital aganglionosis, is a developmental neuropathy where infants are born without an ENS for a variable length of the colon. HSCR occurs at a frequency of 1:5000 live births, and is fatal if left untreated.[10] Gold-standard treatment involves resection of the aganglionic bowel via pull-through surgery, followed by anastomosis of the ganglionated gut to the anal margin. Although this procedure is life-saving, 50% of children continue to experience ongoing symptoms like chronic constipation, incontinence, and enterocolitis.[10] Stem cell therapy has emerged as a promising alternative solution, with the aim of regenerating the missing ENS in the aganglionic gut to restore gut function.[11–14] In this study, we investigated the neurogenic potential of EGCs both in vitro and in vivo following transplantation into a mouse model of HSCR.

Primary EGCs were isolated from the small intestines of *Sox10|ChR2* or *Sox10|GCaMP3* mice, where expression of the genetically encoded light-activated cation channel, *ChR2*, or calcium indicator, *GCaMP3*, is driven by the tamoxifen inducible *Sox10-creERT2* promoter (Supplementary Methods).[15] Of $Sox10^+$ cells, 99.8% ± 0.1% and 95.1% ± 0.5% were found to express ChR2 or GCaMP3, respectively (N = 3 mice for each strain) (Supplementary Figure 1). Reporter expression appeared to be restricted to EGCs, and no co-labeling was identified with HuC/D^+ neurons (N = 3 mice for each strain, n = 3312 HuC/D^+ cells examined). EGCs were cultured as gliospheres, and following the addition of appropriate growth factors in vitro, gliospheres were processed for immunolabeling to investigate their neurogenic potential. To examine in vivo differentiation, gliospheres were transplanted into the distal colon of the $Ednrb^{sl/sl}$ (*Ednrb*-KO) mouse model for HSCR at postnatal day (P)14 to 21. Grafted colons were harvested at P28 to examine neuronal communication through live calcium imaging, ex vivo electrophysiology, and immunolabeling.

To evaluate proliferation, we examined the impact of neuregulin (NRG) and glial-cell line derived neurotrophic factor (GDNF) on gliosphere cultures in vitro. Both growth factors play key roles in ENS development: NRG promotes the proliferation and differentiation of EGCs,[16] whereas GDNF targets both neurons and progenitors, and is a common addition to ENS cultures.[17] We hypothesized that adding NRG to the primary cultures would enhance gliosphere proliferation. Over 2 weeks, GDNF significantly increased sphere size, whereas NRG had no additional significant effect on gliosphere growth (Figure 1*A*). Gliospheres were exposed to 5-ethynyl-2′-deoxyuridine (EdU) for the last 6 hours of culture (Figure 1*B,C*). GDNF alone yielded the highest proportion of EdU^+/4′,6-diamidino-2-phenylindole $(DAPI)^+$ nuclei; NRG did not significantly impact proliferation (Figure 1*B*). Therefore, only GDNF was added to cultures in follow-up experiments.

The neurogenic capacity of gliospheres was first examined in vitro. On average, 19.54% ± 0.92% (n = 4) of $DAPI^+$ nuclei in cultured gliospheres were immunoreactive to the pan-neuronal marker HuC/D, indicating that EGCs had differentiated into enteric neurons (Figure 1*D*). To assess in vivo differentiation, gliospheres were transplanted into the distal colon of *Ednrb*-KO mice. Due to the variable length of aganglionosis in this mouse model, 10 of 16 transplants were identified in the aganglionic region; the rest were located in the transition zone. Green fluorescent protein $(GFP)^+$ cells and processes were observed to migrate out of the graft core, occupying an average area of 700,000 ± 110,000 μm^2 (N = 7 transplanted mice) (Figure 2*A* and *B*). High-magnification images of graft cores and extremities revealed an average of 2 ± 0.2 HuC/D^+ graft-derived neurons per 10,000 μm^2 (N = 7 mice). This density is lower than that of the distal colon in wild-type mice of the same age, which is approximately 10 neurons per 10,000 μm^2 (unpublished data). The lower density of graft-derived neurons is likely due to the short interval between transplantation and tissue harvest, which could not be avoided as our *Ednrb*-KO mice did not survive weaning. Notably, there was substantial variation between the dense graft core and extremities, with most neurons located in the core (Figure 2*A* and *B*). The presence of EdU^+GFP^+ neurons suggests in vivo neuronal differentiation in 4 of 7 transplanted colons (Figure 2*C*); however, it is also likely that many HuC/D^+ neurons in the graft had differentiated during gliosphere culture in vitro. Previous studies have shown that extrinsic excitatory innervation coupled with the absence of local ENS inhibition causes tonic constriction of the aganglionic bowel in HSCR, preventing the propulsion of colonic contents.[18,19] We therefore investigated whether our EGC grafts could help restore enteric inhibitory motor neurons. We found that 29.03% ± 23.06% of HuC/D^+ graft-derived neurons were immunoreactive to the inhibitory marker neuronal nitric oxide synthase (nNOS; weighted mean ± standard error of the mean [SEM] for N = 4 mice) (Figure 2*D*).

Efficient use of EGCs in cell therapy also relies on efficient generation and

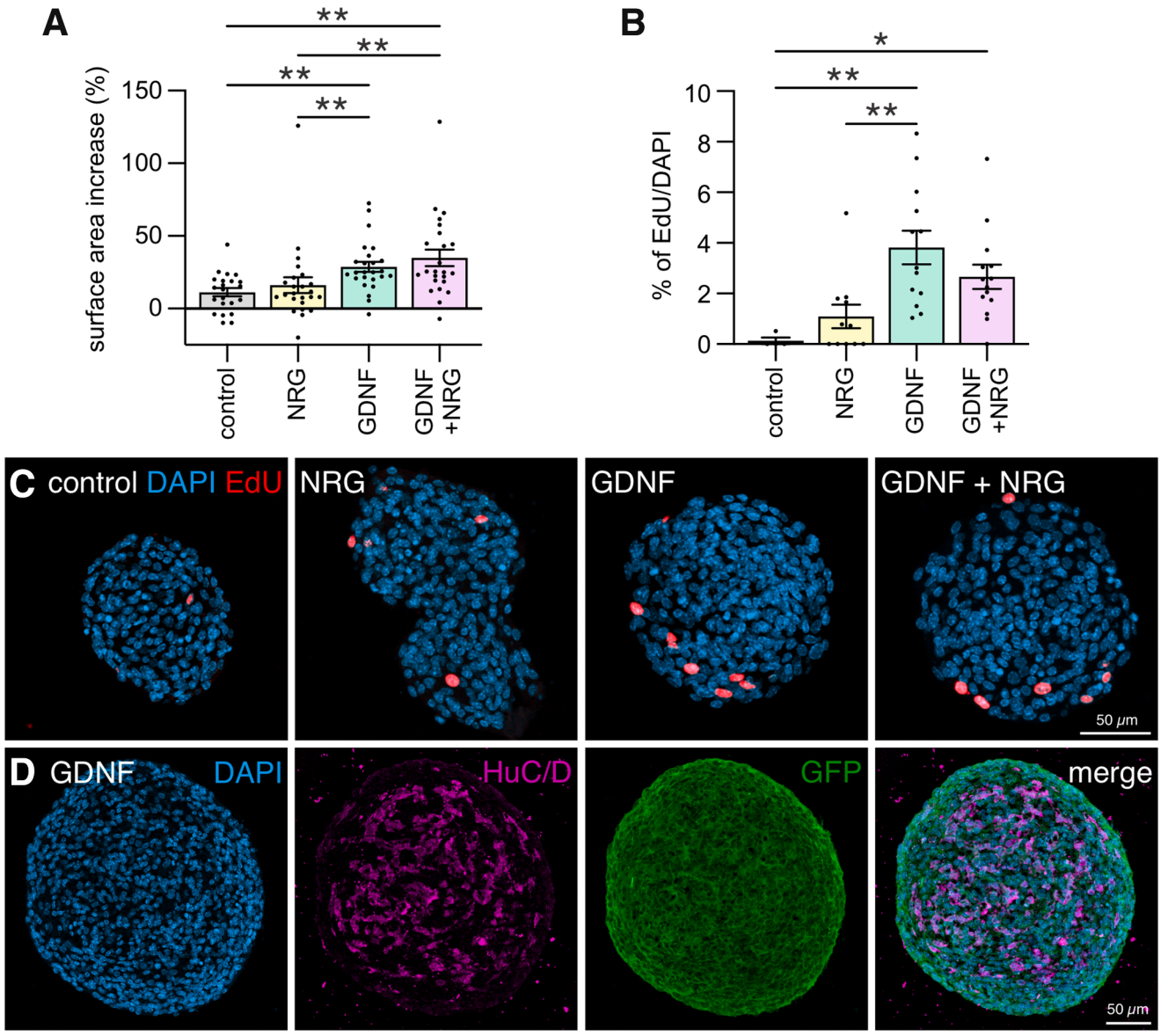
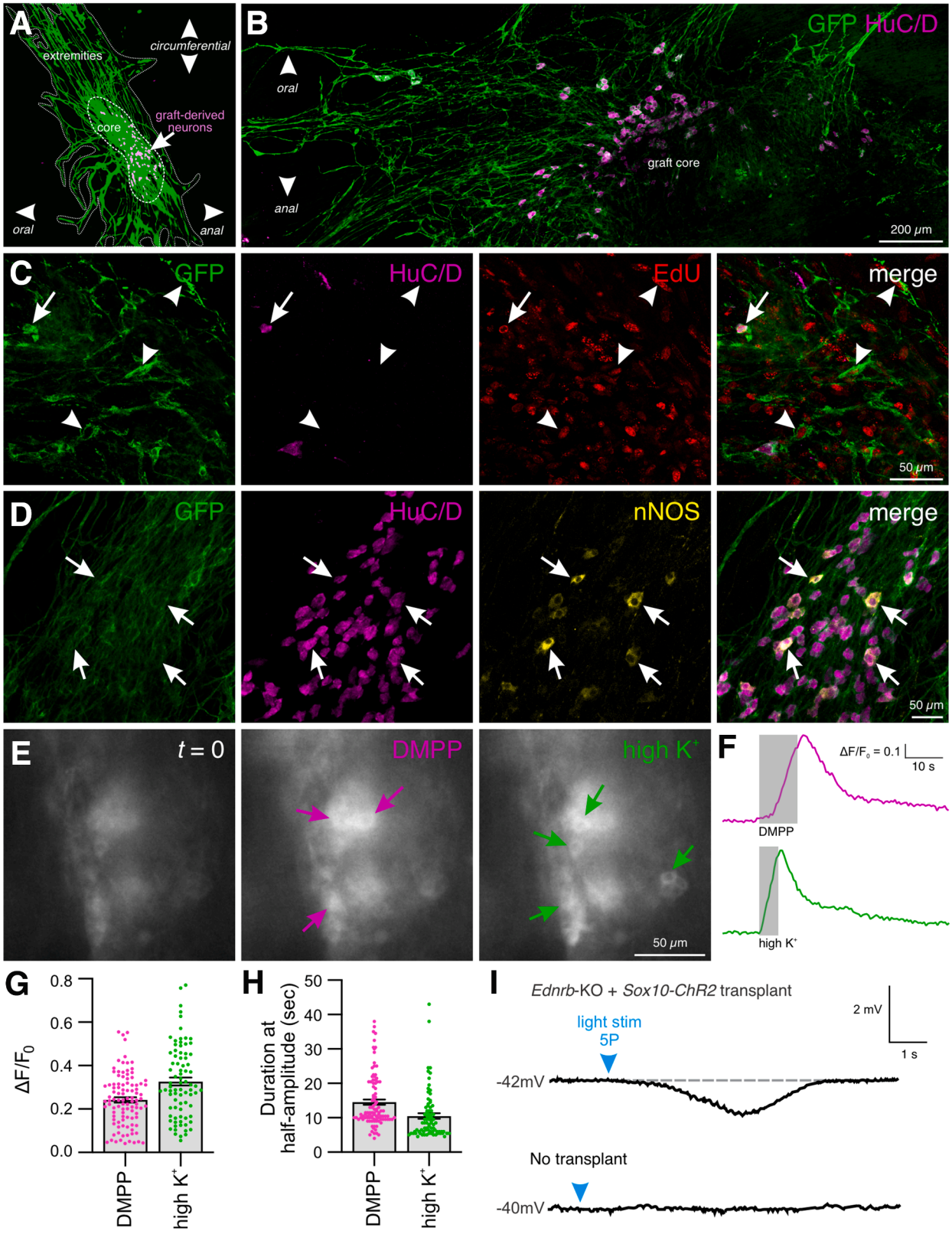


Figure 1. Differentiation and proliferation of enteric gliospheres in vitro. (*A*) Quantification of changes in gliosphere 2D surface area each week in different growth factors. (*B*) Quantification of cell proliferation (% $EdU^+/DAPI^+$ nuclei) in different growth factor conditions. (*C*) Representative images of 5000 cell-gliosphere cryosections in the presence of NRG and GDNF, after exposure to EdU. (*D*) Immunolabeled cryosections of gliospheres (10,000 cell starting population) after 2 weeks in culture with GDNF, using DAPI, anti-HuC/D, and anti-GFP. Data shown as mean ± SEM; with at least n = 4 spheres for each condition and at least 3 cryosections from each sphere. A total of n = 33,336 $DAPI^+$ cells were examined. Kruskal-Wallis test with Dunn's multiple comparison test: $^*P < .05$ and $^{**}P < .01$.

integration of functional neurons into the endogenous gut microenvironment. To test this, live calcium imaging was performed on *Ednrb*-KO mice 1 week after *Sox10|GCaMP3* gliosphere transplantation (Figure 2*E–H*). $GCaMP3^+$ cells responded to application of the nicotinic receptor agonist, 1,1-dimethyl-4-phenylpiperazinium (DMPP), as well as high K^+, which depolarizes both enteric neurons and glia. As nicotinic receptors are not expressed by EGCs, this suggests that gliosphere-derived enteric neurons can be functionally stimulated in the transplant site and are either directly activated by DMPP or receiving inputs from neighboring endogenous neurons.

To examine integration of gliosphere-derived cells with recipient gut smooth muscle cells (SMCs), we first investigated ENS-SMC communication in the ganglionated mid-colon of untransplanted *Ednrb*-KO mice (Supplementary Figure 2). Currently, information on SMC activity in the context of HSCR is limited, with most studies focusing on differences between the aganglionic and ganglionated colon.[20,21] Recent studies have also shown that there are distinct differences in the ENS of the ganglionated colon between *Ednrb*-KO and wild-type mice[22,23]; we therefore investigated whether this translated into changes in ENS-SMC communication. Sharp electrode intracellular recordings were made from SMCs in

the mid-colon, with electrical field stimulation (EFS) and pharmacological antagonists applied to compare the neuromuscular transmission between *Ednrb*-KO and wild-type mice. Although EFS activates both excitatory and inhibitory motor neurons, previous studies have shown that it generally results in hyperpolarizing



inhibitory junction potentials (IJPs) in colonic SMCs, due to a relatively higher contribution of inhibitory neurotransmission.[24] We observed this same effect in the mid-colon of young *Ednrb*-KO and wild-type mice. From each recording, resting membrane potential, as well as electrically evoked IJP amplitude and duration were investigated. None of these parameters were significantly different between genotypes (Supplementary Figure 2*A*). In addition, we were not able to elicit IJPs in the far distal colon of *Ednrb*-KO mice, consistent with the lack of ganglia. To examine the relative contributions of nitrergic and purinergic transmission to SMCs, *N*-nitro-L-arginine (NOLA) was added to inhibit nNOS activity, as well as MRS2179 to block $P2Y_1$ receptors. NOLA significantly reduced IJP amplitude in both genotypes, and the addition of both NOLA and MRS2179 eliminated electrically evoked IJPs, revealing excitatory junction potentials (EJPs). NOLA did not significantly change IJP duration in either *Ednrb*-KO or wild-type mice; however, the addition of both NOLA and MRS2179 on response duration was difficult to determine due to the presence of EJPs (Supplementary Figure 2*B-C*).

Lastly, the communication between transplanted cells and endogenous SMCs was tested via intracellular recordings of SMCs within 3 mm from the gliosphere core. *Ednrb*-KO mice transplanted with *Sox10|ChR2* gliospheres were stimulated with blue light to selectively activate glial-derived cells. Small hyperpolarizing events were recorded in response to light stimulation in 3 of 15 transplanted colons. These light-evoked IJPs were faithfully elicited at the onset of the light stimulus (Figure 2*I*). All 3 responding transplants were located in the aganglionic region. Of these 3 recordings, 2 were from SMCs distal to the gliosphere, and 1 circumferential. We did not observe a clear correlation between the extent of graft integration, neuronal density, and responsive behavior. Light stimulation of untransplanted colons yielded no changes in membrane potential (Figure 2*I*). This demonstrates the potential of glial transplantation to influence the aganglionic colon and highlights the prospect of harnessing patient-derived autologous glial cells for future therapeutic applications. In this study, transplanted *Ednrb*-KO mice could only be maintained for a short length of time following surgery, due to poor survival of the animals upon weaning. Although engraftment of larger populations of enteric gliospheres will ensure better colonization of the aganglionic gut, additional methods to maintain survival of the recipient mice will be required to enable sufficient time for neuronal differentiation and integration. We have previously developed a surgical bypass method in *Ednrb*-KO rats to prolong their survival even without cell transplantation.[25,26] Integration of these techniques will be important to further investigate the functional properties of EGC-derived enteric neurons post-transplantation.

In this proof-of-principle study, we show that enteric gliospheres are able to proliferate, and that, following transplantation into *Ednrb*-KO mice, grafted cells differentiate into functional neurons in vivo and can communicate with the recipient SMCs. Advancing this approach will necessitate strategies to optimize cell migration and colonization, as well as examine the differentiation of transplanted cells into specific neuronal lineages. Overcoming these challenges will pave the way for developing effective cell-based therapies for HSCR, including the generation and use of autologous cells.

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Figure 2. (***See previous page***). **Characterization of gliosphere proliferation, differentiation, and integration after transplantation into the distal colon of *Ednrb*-KO mice.** (*A*) Schematic overview of a representative transplant site. (*B*) Grafted colon region highlighting a *Sox10|ChR2* gliosphere, with cells and fibers immunolabeled with anti-GFP, and graft-derived neurons immunolabeled with anti-HuC/D. (*C*) High-magnification graft images labelled with GFP, HuC/D, and EdU, which was administered post-transplantation. $GFP^+/EdU^+/HuC/D^+$ triple-labeled neurons *(arrow)* were identified in grafts, as well as EdU^+/GFP^+ non-neuronal cells *(arrowheads)*. (*D*) Representative image of graft-derived $nNOS^+$ neurons (*arrows*). (*E*) Ca^{2+} imaging of transplanted *Sox10|GCaMP3* gliospheres showing GCaMP3 fluorescence prior to ($t = 0$) and following stimulation with DMPP and high K^+ Krebs. Responding cells are highlighted by *arrows*. (*F*) Representative $[Ca^{2+}]_i$ traces, with agonist stimulation indicated by shaded bars. (*G* and *H*) Quantification of $[Ca^{2+}]_i$ amplitude and duration (at half-amplitude) in response to DMPP and high K^+ Krebs. Each data point represents an individual cell, with population mean ± SEM. At least 5 recordings were made from at least $N = 3$ transplanted mice. (*I*) Representative traces of intracellular recordings from SMCs in the distal aganglionic colon with and without gliosphere transplant, following blue-light stimulation (5 pulses, each 100 ms duration, 10 mW, 5 Hz) indicated with *blue arrowheads*. Trace of response to blue light stimulation is an average compiled from 3 consecutive recordings.

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Supplementary Material

Note: To access the supplementary material accompanying this article, visit the full text version at https://doi.org/10.1016/j.jcmgh.2025.101693.

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Conflicts of interest
The authors have no conflicts to disclose.

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