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Transplantation of amniotic fluid-derived neural stem cells as a potential novel therapy for Hirschsprung's disease☆,☆☆

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ABSTRACT

Background/Purpose: We have previously shown that embryonic enteric neural stem cells (NSCs) isolated from the intestine colonize aganglionic intestine upon transplantation, but posttransplantation cell survival limits efficacy. The aims of this study were to investigate whether transplantation of amniotic fluid (AF)-derived NSCs could improve survival of the engrafted cells and promote functional recovery of the diseased colon.
Methods: AF cells were induced into NSCs with neurogenic medium, and further differentiated into neurons and glial cells. *Ednrb* knockout mice received an intestinal intramuscular injection of 20,000 AF-derived NSCs into the aganglionic colon. Engrafted cells were visualized and characterized by immunohistochemistry for GFP, neuronal, and glial cell markers. Colonic motility was quantified by colonic bead expulsion time.
Results: AF-derived NSCs had increased expression levels of the NSC marker Nestin and the glial cell marker GFAP compared to enteric NSCs. Transplanted AF-derived NSCs had decreased apoptosis and increased survival compared to enteric NSCs. Colonic motility was significantly improved in *Ednrb* knockout mice transplanted with AF-derived NSCs, as demonstrated by significantly decreased colonic bead expulsion time.
Conclusion: AF-derived NSCs have enhanced survival upon transplantation into a defective enteric nervous system. Transplantation of AF-derived NSCs may represent a potential novel future therapy for the treatment of Hirschsprung's disease.

Hirschsprung's disease is caused by lack of development of migrating neural crest cells in the colon [1]. Currently, there is a lack of effective treatments for Hirschsprung's disease. Even in patients in which the aganglionic region is surgically removed, motility problems commonly persist after surgery [2]. Stem cell transplantation using laboratory cultured neural stem cells (NSCs) to colonize aganglionic intestine and restore intestinal motility has been proposed as a treatment for Hirschsprung's disease [3]. Several potential sources of cells capable of generating enteric neurons have been investigated for enteric nervous system (ENS) replenishment including central nervous system (CNS)-derived NSCs, embryonic enteric neural crest cells and postnatal ENS progenitor cells [4]. Although human fetal CNS tissue or embryonic intestines represent potential sources of human NSCs, ethical controversies persist over the use of these cells. Amniotic fluid (AF), readily available during routine amniocentesis or at the time of delivery, can be easily expanded in culture, and induced into neurogenic lineage NSC [5]. AF-derived stem cells are phenotypically and genetically stable and have a normal karyotype, lack of senescence and retain long telomeres [5,6]. They are nontumorigenic *in vivo* and avoid the ethical complications associated with embryonic stem cells, making them a promising cell source for therapeutic purposes [7]. AF-NSCs have been reported to have a higher expression of neural stemness markers than those of adult stem cells following NSC differentiation [8]. However, evidence that AF-NSC can differentiate into mature and functional enteric neurons to replace lost neurons in the ENS is lacking. The aims of this study were to use an animal model of Hirschsprung's disease to investigate whether transplantation of AF-derived NSCSs would lead to improved survival of the engrafted cells, and enhanced functional recovery of the diseased colon, compared to transplantation of enteric NSCs.

1. Methods

1.1. Induction of AF cells into neural stem cells

AF-derived NSCs were generated using a modification of a previously described method [5,9]. Amniotic fluid was collected from 12.5-day post–coitum embryos of pan-EGFP pregnant C57BL/6 J mice (Jackson Laboratory, Bar Harbour, MA), and the recovered cells were cultured in Dulbecco's modified Eagle's medium/Ham's nutrient mixture F12 (DMEM/F12)/Glutamax/10%FBS (Invitrogen, Carlsbad, CA) containing 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen) at 37 °C in

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5% CO_2. A single-cell suspension was prepared by gentle trypsinization and the cells were incubated with a rabbit anti-c-Kit antibody (CD117) (Invitogen). C-Kit-positive cells were then incubated with magnetic goat anti-rabbit IgG MicroBeads and selected on a Mini-MACS separator apparatus (Miltenyi Biotec Inc., San Diego, CA). The selected AF cells were transferred to a specific NSC induction medium consisting of DMEM/F12 supplemented with 2 mmol/L L-glutamine (Invitrogen), 7.5% (v/v) chick embryo extract (Gemini Bio-products, West Sacramento, CA), 1% (v/v) N_2 medium supplement (Sigma-Aldrich, St Louis, MO), 20 ng/ml mouse basic fibroblast growth factor, and 20 ng/ml mouse epidermal growth factor (Sigma-Aldrich). NSCs grew as free-floating cellular aggregates known as neurosphere-like bodies. To enhance neural differentiation, AF-derived NSCs were switched to a specific neural differentiation medium consisting of neural basal medium (Life Technologies, Grand Island, NY) supplemented with B_{27} (Life Technologies) and 10% FBS.

1.2. Enteric neural stem cell culture

Enteric NSCs were generated as previously described [9]. Briefly, embryonic intestines from 12.5-day post–coitum embryos of pan-EGFP mice were dissected and dissociated in 50 μg/ml dispase and 50 μg/ml collagenase (Worthington Biochemical, Freehold, NJ) for 60 minutes at 37 °C. Cells were cultured in NSC induction medium and neurosphere-like bodies were allowed to form and subjected to repeat passaging.

1.3. NSC transplantation into distal intestine of *Ednrb*$^{-/-}$ mice

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Research Institute at Nationwide Children's Hospital (RINCH) (Protocol # AR11-00088). *Ednrb*^{tm1Ywa} knockout mice on a hybrid C57BL/6J-129Sv background were generated by Taconic Biosciences Inc. (Germantown, NY) and housed in the RINCH vivarium. *Ednrb*^{tm1Ywa} is a targeted null mutation of the endothelin receptor B gene that segregates in an autosomal recessive manner. Homozygous *Ednrb*$^{-/-}$ mice progressively develop megacolon caused by aganglionosis in the distal colon and their life expectancy is from 4 to 5 weeks. In *Ednrb*$^{-/-}$ mice, a 0.5–0.7 cm constrictive segment of the distal gut is present, and the absence of Tuj-1-positive neurons in this segment was confirmed by immunohistochemistry (IHC) of longitudinal sections of the distal gut (data not shown). This aganglionic segment was chosen for intramuscular injection of NSCs. EGFP labeled AF-derived NSCs or enteric NSCs were prepared in a volume of 30 μL PBS. After being anesthetized with isoflurane (Abbot Laboratories, North Chicago, IL), 3-week old *Ednrb*$^{-/-}$ *mice* ($n = 38$) or age matched wild type (*Ednrb*$^{+/+}$) mice ($n = 13$) received a trans-anal intramuscular injection of a cell suspension containing 20,000 NSCs or control normal saline into three sites of the aganglionic distal intestine (12, 4 and 8 o'clock) using a 34-gauge needle. The distal intestine was harvested for histological and biochemical analyses at 3 days or 7 days after NSC transplantation.

1.4. Bead expulsion test as a measure of colonic motility

A modified technique was employed to assess colonic motility [10,11]. After neurotransplantation, animals were subjected to daily anal dilation for 7 days until functional recovery of colonic motility was studied. Mice were lightly anesthetized with isoflurane and a 3-mm petroleum jelly-coated glass bead (Chemglass Life Science, Vineland, NJ) was introduced into the distal colon (2 cm from the rectum) of each mouse to be studied. After bead insertion, mice were placed in individual plastic cages lined with white paper to aid in visualization of bead expulsion. The time required for expulsion of the glass bead was recorded.

1.5. Fluorescence immunohistochemistry (IHC) and immunocytochemistry (ICC)

IHC and ICC were performed as previously reported [12]. Primary antibodies included: chicken anti-Green Fluorescence Protein (GFP) (1 μg/mL) (Invitrogen); mouse anti-Tuj-1 (1:1000; Abcam, Cambridge, MA), rabbit anti-Glial Fibrillary Acid Protein (GFAP) (1:1000; Carpinteria, CA), rabbit anti-cleaved Caspase 3 (1:500; Abcam), and mouse anti-CD68 and anti-CD15 (1:500; Abcam). Secondary antibodies included fluorophore-conjugated goat anti-rabbit IgG (Alexa 488), anti-mouse IgG (Cy3), or anti-rabbit IgG (Alexa 647) as appropriate. For NSC staining, neurospheres were grown in poly-D-lysine/laminin coated slide chambers for 2 days (BD Biosciences, San Jose, CA) and fixed in 4% FPA. Cells were incubated with mouse anti-Rat-401 (Nestin) (1:100; Developmental Studies Hybridoma Bank of the University of Iowa, Iowa City, IA), anti-Tuj-1, or anti-GFAP antibodies, and then incubated with the appropriate secondary antibodies. Fluorescent staining was visualized by confocal fluorescence microscopy.

1.6. Real-time reverse-transcription polymerase chain reaction (RT-PCR)

Cultured NSCs were harvested and total RNA was reverse-transcribed with random hexamers using a first-strand cDNA synthesis kit (Invitrogen-Gibco). Real-time RT-PCR was carried out using a SYBR Green RT-PCR kit (Applied Biosystems, Branchburge, NJ) and an ABI Prism 770 Sequence Detection System (Applied Biosystems). Mouse *Nestin*, microtubule-associated protein 2 (*MAP-2*) and *GFAP* were amplified using the following primers: *Nestin* sense, 5′-GGAACCCAGA GACTGTGGAA-3′ and Nestin anti-sense, 5′-CACATCCTCCCACCTCTGTT-3′; *MAP-2* sense, 5′-CCTTATGGGAATGTGGGATG-3′ and *MAP-2* anti-sense, 5′-AAAAAGTGGGCCTTGGAACT-3′; *GFAP* sense, 5′-AGAAAACC GCATCACCATTC-3′ and *GFAP* anti-sense, 5′-TCACATCACCACGTCC TTGT-3′. Amplification of the housekeeper gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) cDNA was used as an internal control for quantification. Quantification was performed using Relative Quantification Software, version 1.01 (Applied Biosystems).

1.7. Statistical analyses

All data are presented as mean ± SEM. Statistical analyses were performed using the Student *t* test or one-way analysis of variance (SigmaPlot 11.0, Systat Software, Inc. San Jose, CA). $p < 0.05$ was considered statistically significant.

2. Results

2.1. AF-derived NSCs have increased expression of Nestin and GFAP mRNA

Previous reports showed that AF-derived mesenchymal stem cells (MSC) can differentiate into neural precursor cells *in vitro* when exposed to neural induction medium [13]. We have previously shown that AF-derived MSC have increased ability to colonize the intestine compared to other sources of MSC [14], indicating that AF may represent an ideal source of NSCs for transplantation for intestinal neurological disorders, including aganglionic Hirschsprung's disease. We began the current studies by inducing AF-derived MSC to differentiate along a neuronal lineage into NSCs. The specific serum free NSC induction medium we used provides a suboptimal condition for fetal bovine serum (FBS)-dependent MSC growth. After three weeks of culture in serum free NSC induction medium, only NSC colonies are expanded and grow as shown by the formation of typical neurospheres consisting of NSCs or neural precursor cells identified by Nestin immunostaining (Fig. 1A–D). Importantly, AF-derived NSCs further differentiated into neurons and glial cells *in vitro* (Fig. 1E-H). Real time RT-PCR confirmed that AF-derived NSCs had significantly increased expression of Nestin mRNA during the NSC proliferation stage compared to enteric NSCs. In addition, after 2 weeks of culture in neural differentiation medium, AF-derived NSCs had significantly increased expression levels of the glial cell marker GFAP, with equivalent expression of the neuronal marker MAP_2, compared to enteric NSCs (Fig. 1I, J).