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Combination of exogenous cell transplantation and 5-HT_4 receptor agonism induce endogenous enteric neural crest-derived cells in a rat hypoganglionosis model

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ABSTRACT

Enteric neural crest-derived cells (ENCCs) can migrate into endogenous ganglia and differentiate into progeny cells, and have even partially rescued bowel function; however, poor reliability and limited functional recovery after ENCC transplantation have yet to be addressed. Here, we investigated the induction of endogenous ENCCs by combining exogenous ENCC transplantation with a 5-HT_4 receptor agonist mosapride in a rat model of hypoganglionosis, established by benzalkonium chloride treatment. ENCCs, isolated from the gut of newborn rats, were labeled with a lentiviral eGFP reporter. ENCCs and rats were treated with the 5-HT_4 receptor agonist/antagonist. The labeled ENCCs were then transplanted into the muscular layer of benzalkonium chloride-treated colons. At given days post-intervention, colonic tissue samples were removed for histological analysis. ENCCs and neurons were detected by eGFP expression and immunoreactivity to $p75^{NTR}$ and peripherin, respectively. eGFP-positive ENCCs and neurons could survive and maintain levels of fluorescence after transplantation. With longer times post-intervention, the number of peripherin-positive cells gradually increased in all groups. Significantly more peripherin-positive cells were found following ENCCs plus mosapride treatment, compared with the other groups. These results show that exogenous ENCCs combined with the 5-HT_4 receptor agonist effectively induced endogenous ENCCs proliferation and differentiation in a rat hypoganglionosis model.

1. Introduction

Normal activities of the gastrointestinal tract, such as motility, secretion and blood flow, are regulated primarily by the enteric nervous system (ENS) [1], which consists of a compact network of enteric ganglia and their supportive cells. Any disruption to the ENS during embryogenesis can result in enteric defects, such as Hirschsprung's disease, which is characterized by the absence of ganglionic cells and functional bowel obstruction. Transplantation of ENCCs to rebuild or repair the damaged ENS has been investigated as an experimental approach [2]. Although ENCCs can survive, proliferate, migrate and differentiate into progeny cells, including neurons and glia [4–6], and have even partially rescued bowel function [3], limitations remain that need to be solved for efficient clinical application. In particular, the poor survival, migration and differentiation of ENCCs must be addressed. Currently, ENCCs transplantation alone is unable to treat ENS-related disorders; therefore, its combination with other positive treatments must be investigated.

Serotonin (5-hydroxytryptamine, 5-HT) can promote enteric neurogenesis through the 5-HT_4 receptor [7,8]. 5-HT_4 not only functions as a neurotransmitter, but also as an essential growth factor in postnatal growth and maintenance of the ENS [9,10]. 5-HT_4 and its receptor have multiple roles in ENCCs, neurons and the ENS. The addition of the 5-HT_4 or activation of 5-HT_4 receptor increases neuronal numbers and neurite length [11], enhances the plasticity of ENCCs, promotes neuro-differentiation during nerve regeneration [12], and also mediates neuroprotection and neurogenesis in the ENS [7]. A previous *in vivo* study in guinea pigs [13] demonstrated an enhanced intrinsic defecation reflex by stimulation of enteric neural 5-HT_4 receptor with its specific agonist mosapride (MOS). Moreover, a combination of ENCC transplantation and 5-HT_4 receptor activation is predicted to more beneficial than ENCC transplantation alone in

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Fig. 1. Image of surgery and intestinal features of the benzalkonium chloride (BAC)-treated rat model. A schematic diagram of the surgical treatment with BAC (A). The presence of pale colons immediately after surgery (B). Rats had obviously constricted colonic segments (indicated with forceps in C) at the site of BAC treatment, with marked dilatation and dry stool retention proximal to the treated bowel (C). BAC, benzalkonium chloride.

treating ENS-related disorders [14].

Survival of ENCCs is critical to their regulation of the neural network of the gut wall [15,29]; however, transplanted ENCCs can be prone to apoptosis [16,17,18,19]. Our previous study using flow cytometry also revealed apoptosis of ENCCs during their culture *in vitro* [20]. In addition to apoptosis, the lack of nutritional supports (*i.e.* growth factors), physical damage, immune rejection and free radicals, as well as the intestinal microenvironment, limit the successful transplantation of ENCCs [21,22].

Endogenous ENCCs exist in the ENS of humans and other mammals and can be derived from adult and even aganglionic ENS [23]. Thus, the mobilization and recruitment of endogenous ENCCs in the ENS could promote nerve regeneration. Exogenous ENCCs transplantation treated with MOS could, therefore, serve as a novel therapy to rescue functional bowel obstruction.

This study used multi-point injections of labeled ENCCs into the colon of hypoganglionosis model rats treated with MOS, and observed the survival, proliferation, and differentiation of exogenous and endogenous ENCCs. We also analyzed the effects of this combination treatment on endogenous ENCCs. This combination strategy may provide a novel therapy for the repair of the injured ENS.

2. Materials and methods

2.1. Experimental animal preparation

ENCCs were harvested from one-day-old Sprague-Dawley (SD) rats. Adult female SD rats, body weight at 56 days, were used to prepare the hypoganglionosis animal model. Animals were obtained from the Center of Experimental Animals, College of Medicine, Xi'an Jiaotong University, China. Animals were handled in accordance with the guidelines outlined by the Animal Care and Ethics Committee of Xi'an Jiaotong University and the National Institute of Health Guide to the Care and Use of Laboratory Animals.

2.2. ENCCs culture and sub-culture

All procedures were performed under aseptic condition as described previously [20]. Briefly, newborn rats were sacrificed by quick cervical dislocation. The outer muscular layers of gut samples were carefully removed under a stereomicroscope. The tissue was dissociated by repeated trituration, followed by incubation in 0.25% trypsin at 37 °C for 12 min. Samples were then centrifuged at 800 rpm for 5 min at room temperature to collect single-cell suspension of ENCCs. The pelleted ENCCs were then resuspended in DMEM F12 (1:1) (SH30023.01B; Life Sciences, USA), supplemented with N2 (0.5%, 17502-048; Gibco, USA), B27 (1.0%, 12587-010; Gibco), recombinant fibroblast growth factor-basic (bFGF, 10 ng/ml, PHG0021; Gibco), recombinant epidermal growth factor (EGF, 10 ng/ml, PHG0311; Gibco), 2-mercaptoethanol (50 μmol/l, M3148; Gibco), penicillin and streptomycin (100 U/ml, 15140148; Gibco) at a density of 5×10^5 cells ml-1, and maintained at 37 °C in water-saturated 5% CO_2 incubator. The floating cells, constituting ENCCs, were harvested and supplemented with fresh media. The number of cells was determined using a hemocytometer. Medium was replaced every 24 h until the cells were used for experiments. Neurospheres from single ENCCs were dissociated into single ENCCs, and then re-cultured by the same method to obtain sub-cultures. To induce ENCC differentiation, bFGF and EGF were omitted and fetal calf serum (1.0%, 10099-141; Gibco) was added.

2.3. Immunohistochemistry

Immunohistochemistry was performed on paraffin sections and cells as previously described [20,24,25]. The following specific antibodies were used for immunohistochemistry to detect ENCCs ($p75^{NTR}$, ab104576, Abcam, UK; nestin, ab92391, Abcam, UK), neurons (peripherin, ab4666, Abcam, UK; β-tubulin, ab52866, Abcam, UK), and glial cells (glial fibrillary acidic protein, GFAP, AF2594, Thermo Fisher, Waltham, Massachusetts, USA). Cell nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI, 10236276001, Roche, Germany).

2.4. Establishment of hypoganglionosis rat model

All surgical procedures used in this study have been previously described [24,25]. Under anesthesia by intraperitoneal injection of 10% chloral hydrate solution, 1-cm segments of the descending colon adjacent to the bladder were exteriorized through a midline incision, wrapped tightly with filter paper and soaked thoroughly every 3 min for 30 min with 0.1% benzalkonium chloride (BAC) (Fig. 1). After incubation, the BAC-soaked filter paper was removed and the treated segments were thoroughly washed several times with tepid saline, returned to the abdominal cavity, and the abdomen was closed using silk sutures. The rats were socially housed and fed with standard diet and tap water ad libitum for 48 h postoperatively. Hematoxylin and eosin (H & E) and immunohistochemical staining were performed to evaluate the myenteric plexus at 4 weeks after BAC treatment. H & E staining and immunohistochemistry were applied to count the number of ganglia and ganglionic cells per section in a 1-cm ×5-μm slice.

2.5. Labeling of ENCCs

Lentivirus produced from the plasmid vector pLOV.CMV.eGFP.2A.EF1a.PuroR and packed by human embryonic kidney 293 T cells, was obtained from NeuronBiotech (HYXH-lenti-210026; NeuronBiotech, Shijingshan, Beijing, China). The lentivirus protocols used have been described previously [20]. Briefly, ENCCs were subcultured in 24-well plates at 1×10^4 cells/well for 24 h. Lentivirus was added to the wells at 25× multiplicity of infection for 24 h. Forty-eight hours after transduction, fluorescence-activated cell