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***In vitro*-conditioned bone marrow-derived mesenchymal stem cells (BMSC) promote *de novo* functional enteric nerve regeneration, but not through direct-transdifferentiation**

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Abstract

Injury or neurodegenerative disorders of the enteric nervous system (ENS) cause gastrointestinal dysfunctions for which there is no effective therapy. This study, using the BAC-induced rat gastric denervation model, aimed to determine whether transplantation of bone marrow-derived mesenchymal stem cells (BMSC) could promote ENS neuron regeneration and if so, to elucidate the mechanism. Fluorescently-labeled BMSC, isolated from either WT [BMSC labeled with bis-benzimide (BBM)] or GFP-transgenic rats, were preconditioned *in vitro* using fetal gut culture media containing glial cell derived neurotrophic factor (GDNF), and transplanted subserosally into the denervated area of rat pylorus. In the nerve-ablated pylorus, grafted BMSC survived and migrated from the subserosa to the submucosa 28 days after transplantation, without apparent dedifferentiation. A massive number of PGP9.5/NSE/HuC/D/Tuj1-positive (but GFP- and BBM-negative) neurons were effectively regenerated in denervated pylorus grafted with preconditioned BMSC, suggesting that they were regenerated *de novo*, not originating from trans-differentiation of the transplanted BMSC. BMSC transplantation restored both basal pyloric contractility and EFS-induced relaxation. High levels of GDNF were induced in both *in vitro*-preconditioned BMSC as well as the previously denervated pylorus after transplantation of preconditioned BMSC. Thus, a BMSC-initiated GDNF-positive feedback mechanism is suggested to promote neuron regeneration and growth. In summary, we have demonstrated that allogeneically transplanted

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Authorship contributions: RL and ZD designed/performed most of the experiments/data analysis, and wrote the manuscript; HM, HS, YG, WQ, and WS helped in some of the experiments; ZL provided technical assistance in animal surgery; XH was involved in project design and supervision; XL was involved in project design, supervision, and manuscript revision

preconditioned BMSC initiate *de novo* regeneration of gastric neuronal cells/structures that in turn restore gastric contractility in pylorus-denervated rats. These neuronal structures did not originate from the grafted BMSC. Our data suggest that preconditioned allogeneic BMSC may have therapeutic value in treating enteric nerve disorders.

Keywords

Bone marrow stromal cells; stem cell transplantation; bone marrow-derived mesenchymal stem cell (BMSC); tissue regeneration; neural differentiation neural induction; Neural stem cell

INTRODUCTION

The enteric nervous system (ENS) is a part of the peripheral nervous system (PNS) that operates largely independently of central nervous system (CNS) to coordinate the complex behaviors of gastrointestinal (GI) tract. ENS abnormality or dysfunction can lead to dysmotility syndromes include achalasia, gastro-esophageal reflux disease, delayed emptying of the stomach, abdominal pain and bloating, diarrhea and constipation[1]. Besides congenital motility disorders of ENS [eg Hirschsprung disease (HSCR)[2]], neuron degeneration also occurs in a large number of other diseases or pathophysiological conditions, such as diabetic gastroparesis, intestinal pseudo-obstruction of motility, and age-related neuronal loss in ENS[3]. Remarkably, recent reports have shown that lesions in ENS occur at a very early stages of these diseases, even before the involvement of the central nerve system [4]. So far, no effective therapy is available for these syndromes or disorders, and thus new and effective treatments are urgently needed[1,3,5].

Advances in stem cell research over the past two decades have opened up the possibility of using stem cells to treat neuron injury or degeneration diseases [6,7,8]. Multiple types of stem cells, including embryonic stem cells (ESC)[9], ESC-derived neural precursors[10], induced pluripotent stem cells (iPSC)[11], neural stem cells[12], neural crest-derived stem cell[13], enteric nervous system stem cells[14,15], have been demonstrated to be capable of being converted to neural and glial lineage [6,7,16,17]. Adult bone marrow-derived mesenchymal stem cells (BMSC) are multipotent progenitors that are capable of osteogenic, adipogenic and chondrogenic differentiation, as well as displaying transdifferentiation potential beyond the mesenchymal lineages, including differentiation into neurons (although this remains controversial) [18,19,20,21,22,23]. In addition, due to their active expansion capacity, high plasticity, and especially their low immunogenicity, BMSC remain as unique and attractive candidates for allogeneic cell-replacement therapies[23,24].

Functional improvement through BMSC therapy has been reported in animal models of CNS injury, such as traumatic brain injury or spinal cord injury et al [8,25,26,27,28,29]. While some of the underlying mechanisms have been well documented, others are controversial or being challenged or poorly understood, including involvements of direct-transdifferentiation of BMSC into neurons, spontaneous cell fusion, anti-inflammatory property and modulation of neurotrophic mediator[18,19,30,31,32]. In addition, it is unclear how BMSC take part in ENS circuit repair[33]. BMSC have been shown to home to the stomach for injury repair during chronic gastric inflammation [34]. It has been demonstrated that a 5-HT₄ receptor

agonist could induce *in vivo* neurogenesis in a rectal anastomosis model. This neurogenesis was mediated through enteric neural stem cells possibly from neural crest-derived stem cells or BMSC [35,36], suggesting a possible contribution of BMSC in ENS restoration.

The properties and clinical applications of BMSC have always been a focus of debate since data from different reports are not always consistent, most likely due to the lack of disease-specific standardization or less-than-optimal experimental conditions [18,19,20,21]. For these reasons, MSC researchers have made significant efforts, by modulating microenvironments of MSCs (“reconditioning” or “reprogramming” of MSC) before and after transplantation, on improving therapeutic potential and consistency[37,38,39].

To promote the potential or effectiveness of BMSC in neurogenesis in GI tract, in this report, we preconditioned BMSC with GDNF and fetal gut extracts to mimick the growth microenvironment of fetal neurons. We demonstrate here that intra-pyloric transplantation of *in vitro* preconditioned neuron-like BMSC effectively induces massive *de novo* regeneration of neurons and improvement of gastric motility of myenteric plexus-ablated rats. Intriguingly, these regenerated neurons did not originate from the grafted BMSC, suggesting that the gastric neuron regeneration was induced by grafted preconditioned BMSC but not from direct BMSC transdifferentiation. A GDNF positive feedback mechanism of BMSC is hypothesized to be involved in the processes of *de novo* regeneration of new neurons.

MATERIALS AND METHODS

Animal

Adult Sprague-Dawley rats (SD rats; 10-12 week old), either WT or GFP transgenic), were used in this study. Animal were maintained and used in accordance with NIH guidelines. Animal study protocols were approved by the Animal Care and Use Committee (ACUC) at the Johns Hopkins University and Huazhong University of Science & Technology (HUST). Animals were maintained in either the BRB Animal Facility at the Johns Hopkins University School of Medicine or in the Experiment Animal Facility of HUST, under controlled temperature (25°C) and photoperiods (12h:12h light-dark cycle). Adult male WT or GFP rats (10-12 week old) were used as donors of BMSC. The adult female SD rats were used to establish the pyloric denervation model and also as recipients of BMSC transplantation.

Preparation and characterization of BMSC

BMSC isolated from femurs were cultured in modified eagle medium (MEM) containing lower concentration of glucose (1,000 mg/L), 15% fetal bovine serum (FBS), 2 mM glutamine, 100U/ml penicillin and 100µg/ml streptomycin. 24 h after plating, non-adherent cells were removed by replacing the medium. The BMSC were split (0.25% trypsin/1 mM EDTA) and further enriched by passage cultures. BMSC at passage 6 were stained with antibodies to CD73, CD90, CD105 and CD45 (eBioscience, San Diego, CA, USA) and characterized by FACS analyses. FACS analyses are detailed in the Supplementary Data Methods Section.

***In vitro* adipogenic, osteogenic and chondrogenic differentiation of BMSC**

Detailed *in vitro* differentiation of BMSC into adipocytes, osteoblasts, and chondrocytes are described in Supplementary Data Methods Section.

***In vitro* differentiation of BMSC by glial cell-derived neurotrophic factor (GDNF) in fetal gut culture medium (FGCM)**

After passage 6, BMSC exhibited a distinct spindle-shaped morphology and a continuous expansion potential in *in vitro*-culture (Supplemental Fig. 1). The BMSC in P6 were first pre-induced with basic fibroblast growth factor (bFGF, 10 ng/ml) in DMEM medium for 24 hrs. For *in vitro* differentiation, the pre-induced BMSC were incubated in DMEM medium containing GDNF (10 ng/ml) and FGCM for 10 days, with medium change every 3 days. The FGCM solution was prepared as described [40] with modifications. Controls were BMSC that were cultured in DMEM only (no GDNF or FGCM). BMSC cultured in the absence of GDNF and FGCM did not undergo differentiation. The detailed protocol is described in the Methods section of Supplemental Data.

Establishment of pyloric denervation model using benzalkonium chloride (BAC)

The submucosal and myenteric plexus in rat pylorus was ablated using a myenterically and extrinsically denervated rat model [41,42] with modifications. Rats were anesthetized with 45 mg/kg sodium pentobarbital and the pylorus was exteriorized through a 1-cm midline incision. Patches of gauze pre-absorbed in three different concentrations of BAC (0.1%, 0.3%, and 0.5%) were circularly applied, respectively, to the entire serosal surface of 3-cm segments that span the pyloric region for 20 minutes (1.5 cm each at proximal duodenum and distal antrum; see the schematic illustration in Fig. 2A). The 1-cm middle segment of the denervated area was defined as the regions of interests (ROI), as illustrated in Fig. 2A. All experimental analyses, including efficiency of denervation or BMSC-initiated regeneration, were restricted to the ROI. Sham control rats were treated identically except that the gauze was soaked with 0.9% saline (instead of BAC). The ROI specimens from BAC-treated and saline-treated sham-operation group were dissected at day 1, 7, and 28 post-transplantation, respectively (see below). There were 4-6 rats each group, including treatments with various concentrations of BAC or sham-operation.

Transplantation of BMSC

For transplantation, *in vitro* preconditioned PGP9.5-positive BMSC, or unconditioned PGP9.5-negative BMSC (controls) were fluorescently labeled by incubating with 1 µg/ml bis-benzimide (BBM; Hoechst 33258, Sigma) for 24 hours. The BBM-labeling allows tracking the transplanted BMSC *in vivo*. Before transplantation, for the BMSC that labeled with BBM, cells were washed with DMEM three times to remove the excessive BBM. BMSC generated from bone marrow of GFP-transgenic rats were also used as the second approach to track the BMSC after transplantation.

BMSC (BBM- or GFP-labeled) were prepared at a concentration of 10,000 cells/µl in Dulbecco's phosphate-buffered saline (PBS) and kept on ice. Three days post-BAC treated denervation, 50 µl of BMSC suspension was injected, twice (each on the opposite side of the pylorus) from serosal surface into the longitudinal smooth muscle of denervated pylorus

using a 22 -gauge needle attached to a 50- μ l Hamilton syringe (a total of 1.0×10^6 cells were injected per rat). Same volume of DMEM was given in the same way to the shame operation group. The experimental BAC-treated rats were divided randomly into 4 groups: preconditioned BMSC transplantation group (n=15), sham operation group (n=15), unconditioned BMSC transplantation group (n=5), and preconditioned GFP-BMSC transplantation group (n=5).

Measurement of isometric contractility

Muscle strips isolated from pylorus containing the denervated zone were prepared as we previously described [43]. Tension under basal condition and relaxation under electric field stimulation (EFS) condition were measured according to a previously described protocol [44]. Results are mean $\pm$ SEM from 8–11 strip preparations (from 8–11 rats) for each group.

Real-time (RT)-PCR, Western blotting and Immunohistological analysis were performed as previously described[45,46], as well as described in Supplementary Materials and Methods session.

Statistical analysis

Quantitative expression of proteins/mRNAs of interests was calculated as the ratio of integrated optical density of protein or mRNA to that of β -actin. Quantification of mRNA or protein expression was calculated from at least 10 samples in three independent experiments. Results are shown as mean $\pm$ SEM. All statistical analyses were carried out using SPSS software (Version 11.5; SPSS; Chicago, IL, USA) with one-tailed Student's t-test. Statistical significance was set at $p < 0.05$.

RESULTS

Generation of highly homogenous CD90/CD73/CD105-positive and CD45-negative rat BMSC with multipotent differentiation potential

Bone marrow-derived adherent cells were isolated and cultured as described in the Methods, as we reported previously[47]. 24h after seeding, BMSC isolated from rat bone marrow (see Supplemental data) adhered to the bottom of plastic flasks. The cells grew slowly as single colonies 3–7 days post-seeding (Supplemental Fig. S1A, a–c). During P2 to P6, BMSC became characteristically spindle-shaped (Supplemental Fig. S1A, d–f). BMSC were capable of efficient cell expansion for at least 20 passages (the most we tested). Flow cytometry analysis of P6 demonstrated that the BMSC were positive with BMSC surface antigen CD90 (99.70%-positive), CD73, CD105 (95.9%-positive), while negative for hematopoietic cell marker CD45 (Supplemental Fig. S1B). These cells are able to differentiate to osteoblasts, adipocytes and chondroblasts under standard *in vitro* differentiating conditions (Supplemental Fig. S2).

Isolated BMSC were efficiently preconditioned *in vitro* into neuron-like cells in GDNF-containing fetal gut culture medium (FGCM)

In order to differentiate the isolated BMSC into a neuron-like phenotype (expressing multiple neuronal markers) prior to transplantation, isolated BMSC were preconditioned

with basic fibroblast growth factor (bFGF) and glial cell line-derived neurotrophic factor (GDNF) in FGCM. The expression of PGP9.5 and nNOS was essentially undetectable in unconditioned BMSC (Supplemental Fig. S1C). However, the majority of the preconditioned BMSC abundantly expressed the well-established neuronal markers, including PGP9.5 ($80.3 \pm 3.1\%$), NSE ($79.3 \pm 10.3\%$), Tuj1 ($75.1 \pm 8.2\%$), HuC/D ($72.7 \pm 7.4\%$). These data indicate that preconditioned BMSC exhibit a neuronal phenotype under our induction conditions (Fig. 1). In addition, $78.3 \pm 5.3\%$ of preconditioned BMSC also expressed neuronal nitric oxide synthase (nNOS). nNOS could produce nitric oxide, a major inhibitory neurotransmitter in the ENS. The expression of nNOS in these preconditioned BMSC further indicates their “neuron-like” phenotype (Fig. 1).

Higher concentration of benzalkonium chloride (BAC) is necessary for denervation in pylorus

To ensure complete BAC-induced denervation, a region of interest (ROI, a 1 cm area in the middle of the 3 cm BAC-induced denervated region of pylorus), was studied (Fig. 2A). The pylorus was also used as an anatomical structure for transplanting BMSC to the area where denervation occurred. Initial experiments were directed at determining the optimal dose of BAC (0.1%, 0.3%, 0.5%) for pylorus denervation as shown in Supplemental Fig. S3. 28 days after 0.1% and 0.3% BAC treatment, PGP9.5 positive neurons were significantly reduced, but still detectable. However, in rats treated with 0.5% BAC, PGP9.5 positive neurons became undetectable with either IF or IHC (Fig. 2B). These observations were further confirmed by Western blot analyses of PGP9.5 expression, which was markedly decreased (to an essentially undetectable level) in the ROI of pyloric wall after 0.5% BAC treatment [Fig. 2C (2.28 ± 0.28 vs. 0.19 ± 0.06 ; $p < 0.01$)]. The data indicate that application of a higher concentration BAC (0.5%) to a longer segment of pylorus (an additional 1 cm at each side of ROI) is sufficient to induce and maintain a virtually complete denervation in the ROI for at least 28 days (Fig. 2 and Supplemental Fig. S3).

Allogeneic preconditioned BMSC not only survive in the niche of pyloric wall but also maintain a neuron-like phenotype 28 days after transplantation

To determine the success of BMSC transplantation and new nerve development, at various post-transplantation time points, the ROI of pyloric wall was dissected and then cut into serial sections and analyzed. Survivability and localization of grafted BMSC, as well as the localization of new neurons were carefully examined and compared by a blinded observer.

On day one, transplanted BMSC, administered by sub-serosal injection, were observed only at the serosal or sub-serosal locations (Fig. 3A, left panel). 7 days after transplantation, most BMSC had migrated to the longitudinal/circular muscle layer (Fig. 3A, middle panel). At day 28, transplanted cells had survived and accumulated predominantly in the submucosa (blue, Fig. 3B-a). It is clear that the density of grafted BMSC (stained as blue color by BBM) had shifted their location from the serosal side toward the mucosal layer over time.

Most transplanted cells retained their neuron phenotype (PGP9.5/Tuj1/HuC/D/nNOS-positive) as shown in Fig. 3B-d,f, the merged images of Fig. 3B-a & b) [purple or pinkish purple as the result of merging blue (BBM) and red (PGP9.5)]. Only a small number of

grafted BMSC exhibited PGP9.5-negative phenotype (blue color only in merged image, Fig. 3B-d,e). Interestingly however, these negative cells were localized close to the mucosa layer, Fig. 3B-c,d,e; blue color), but not that of the submucosa (Fig. 3B-c,d,f, blue).

Regenerated myenteric neuronal structures do not originate from transplanted BMSC

28 days after transplantation of preconditioned BMSC, a massive number of new neurons or neuronal structures were regenerated in each rat (Fig. 3B and Fig. 4A, red). This did not occur in rats transplanted with unconditioned BMSC (Supplemental Fig S4). These regenerated neuronal structures exhibited multiple neuronal markers, including PGP9.5, Tuj1, HuC/D and nNOS (immunohistology: Fig 3B, Fig. 4A and Fig. 5, red; Western blots: Fig. 4C). Most of these newly regenerated neuronal structures were localized in the muscular layer, especially in circular muscle and the space between the longitudinal and circular muscular layers (Fig. 3B and Fig. 4A; red). Compared with normal control rats (sham controls, no BAC treatment), regenerated PGP9.5-positive neuronal structures in the muscular layer are less ordered (Fig. 4B). The regenerated neurons exhibited various morphological features (shapes and sizes) (Supplemental Fig. S5). BMM-labeled blue fluorescence could not be detected in these regenerated neuron-like structures (Fig. 3B and Fig. 4A; red and blue), indicating that these structures had not originated from the grafted BMSC. This result was further confirmed by the observation from the transplantation of GFP-BMSC. The newborn PGP9.5-positive neurons did not co-localize with grafted GFP-BMSC (Fig. 5).

BMSC transplantation induced a dramatic increase in the expression of multiple neuronal markers

Consistent with immunohistology (Figs. 3 and 4), Western blot analyses (Fig. 4C) showed a marked increase in the expression of three neuronal markers (PGP9.5, NSE, Tuj1) and nNOS in the pylorus of denervated rats 28 days after transplantation of *in vitro* preconditioned BMSC, when compared to those in BAC-treated sham controls. Statistical analysis demonstrated at least a 3-fold increase in the expression of PGP9.5, NSE, Tuj1 and nNOS (Fig. 4C, low panel). Also consistent with the immunohistological data, the transplantation with unconditioned BMSC led to little change in the expression of these neuronal proteins compared to that with preconditioned BMSC (Supplemental Fig S6).

BMSC transplantation markedly restores the EFS-induced relaxation in denervated pylorus

Muscle strips from BAC-ablated rats exhibited increased tension and irregular peaks, when compared to those from control (sham) animals. BMSC transplantation significantly corrected these abnormalities, including both tracing pattern (Fig. 6A,B) and contraction tension (Fig. 6C,D: sham operation *vs.* BAC-denervated rats with vehicle alone *vs.* denervated rats with BMSC-transplantation = 0.19 ± 0.01 g *vs.* 3.35 ± 0.21 g *vs.* 0.55 ± 0.04 g, $p < 0.01$). Specifically, as shown in the representative tracing in responses to increasing frequencies of stimulation (1 – 8 Hz) (Fig. 6C), EFS-induced frequency-dependent relaxation of the circular pyloric muscle strips isolated from sham group was abolished after BAC treatment. However, in BAC-denervated rats transplanted with preconditioned BMSC, the EFS-induced relaxation was markedly improved (increased), when compared to that from sham BAC-denervated rats (Fig. 6C,D $p < 0.01$) (or group receiving unconditioned

BMSC, data not shown). The NOS inhibitor L-NAME completely blocked this restoration (Fig. 6E).

“GDNF positive feedback” phenomenon was observed both *in vitro* and *in vivo*

As a neurotrophic growth factor, GDNF plays an important role in the development of the ENS. In rats grafted with *in vitro* preconditioned BMSC, we observed a potential positive feedback mechanism of endogenous GDNF expression. BMSC endogenously expressed GDNF protein at very low level, however a significantly increased level of endogenous GDNF expression in BMSC was detected after *in vitro* precondition by exogenous GDNF [0.23 ± 0.01 vs. 0.09 ± 0.01 (mean $\pm$ SEM), Fig. 7A). Furthermore, *in vitro* preconditioned BMSC induced a sustained and stably high level of *in vivo* GDNF expression in transplanted rats for 28 days, compared to sham controls (Fig. 7B). This “GDNF positive feedback” model is illustrated in Supplemental Fig. S7 (see details in Discussion).

DISCUSSION

We present here the first evidence that allogeneic *in vitro*-preconditioned BMSC are capable of effectively inducing regeneration of enteric neurons and restoring BAC-induced defective gastric contractility. The allogeneic preconditioned BMSC survived and maintained a neuronal phenotype (strongly positive in neuronal markers) in ablated pylorus for at least 28 days in the absence of an immunosuppressive agent or caspase inhibitors. Importantly, the regenerated neurons did not originate from the transplanted BMSC.

Applying BAC to the serosal surface of the gut is a common method to chemically generate an experimental model of ENS denervation[41,42,48]. In 2003, Hanani et al described the regeneration of nerve fibers and neurons in the BAC-treated area which were thought to have originated from myenteric neurons adjacent to the denervated region[49]. However, it was recently reported by Joseph et al, using BrdU incorporation, that no neuron regeneration occurred in the ablated region even months after BAC treatment[50]. To eliminate the possibility raised by Hanani, we extended the length of denervation segment around the ROI (Fig. 2A). We found that serosal application of 0.5% BAC for 20 minutes to a 3-cm segment led to sufficient and highly reproducible pyloric denervation in the ROI for at least 28 days (Fig. 2).

The current knowledge regarding the function of BMSC in ENS neurogenesis is very limited. It was suggested that messages released after damage to the ENS or loss of neurons, could direct the migration of stem cells into ENS ganglia [35,36,51,52]. It was also speculated that BMSC were involved in mucosa-derived neurosphere-like body formation[6,14]. Although no consensus or clear mechanism was revealed, most, if not all, recent reports had suggested a role of BMSC in the modulation of ENS regeneration.

Moreover, BMSC have long been considered as a uniquely promising tool for allogeneic cell therapy based on their easy accessibility, active cell expansion capacity, high plasticity, and especially their immune privilege during the allogeneic transplantation. Post-transplantation survival appears to be a critical limiting factor for successful anatomical and functional repopulation in the host tissue[7,8,18,53]. Since we show that allogeneic BMSC survived in

a niche for at least 28 days in the absence of immunosuppressive agents or caspase inhibitors, our study demonstrates a significant practical benefit toward development of BMSC-based therapy on ENS disorders.

Compared to a previous report[54], we achieved a higher efficiency of BMSC transition into “neuron-like state” as a result of our modified *in vitro* pre-conditioning protocol (Fig. 1,3 &4). Phenotype maintenance of *in vitro* preconditioned BMSC has been considered difficult, most likely due to transdifferentiation[18,19,22,54]. The transdifferentiation ability of BMSC presents a significant challenge to the development of BMSC-based therapy as it may occur as a consequence of a stress response. In our study, we found that the preconditioned BMSC maintained a PGP9.5/Tuj1/HuC/D/nNOS-positive phenotype in the submucosa (predominantly) and muscular layer for at least 28 days. Both BMSC migratory behavior and phenotype indicated that the submucosal layer is the most suitable niche for grafted BMSC.

We demonstrated a highly effective regeneration of neurons or neuronal structures in submucosa and particularly the muscle layer by grafted preconditioned BMSC, but not unconditioned BMSC. This result provides strong evidence that preconditioned BMSC initiated the neurogenesis. These data strongly suggests that efficient pre-differentiation of BMSC by GDNF/FGCM *in vitro* before transplantation is absolutely essential as an early “priming” or “pre-conditioning” step for neuron regeneration in the denervation model. However, these regenerated neuron-like cells/structures did not originate from grafted BMSC, since they were negative in both fluorescence BBM and GFP (Figs. 3, 4, and 5).

It is unknown where and how these regenerated neuron-like cells/structures originate. One potential possibility is that grafted BMSC initiated *de novo* regeneration of new neurons by perhaps attracting local or distant migrating stem cells to the muscle layer. We hypothesize that *in vitro* preconditioned to PGP9.5-positive grafted BMSC may create a “transient niche” at the denervated site by secreting specific signaling molecules or factors. This specific niche “attracts” and then activates the proliferation and differentiation of indwelling or migrating stem cells, leading to *de novo* neuron regeneration. Recently, Mathur et al has reported that adult midgut progenitors could generate transient niches that determined the intestinal stem cell fate[55]. On the other hand, the evidence presented could not completely exclude the possibility that the reappearance of neuronal staining was not from neighboring neuron axons; it was likely that the “transient niche” created by preconditioned BMSC encouraged growth of neighboring neuron axons into the denervated region through the expression of GDNF or other neuronal factors, and the unconditioned BMSC could not.

To further explore the mechanisms, we analyzed multiple neurotropic factors secreted by grafted BMSC. These could potentially be the BMSC-derived specific factors that contribute to the unique niches and initiate ENS regeneration. GDNF not only functions as a neuron protective factor, but more importantly as an essential factor for the development of the ENS[2,56]. Based on our data, we propose a “BMSC-initiated GDNF positive-feedback” mechanism, as illustrated in Supplemental Fig. S7, that at least partially mediates the initiation and expansion of new neurons. A similar phenomenon was reported in kidney-derived MSC[57]. Transient increase of GDNF expression in acute injury was previously

reported [58,59], started within 30 min after injury, peaked within 3 h, and then decreased rapidly after 24 h. We demonstrated that a high level of GDNF was expressed *in vivo* 28 days post-transplantation in the denervated region of rats transplanted with preconditioned BMSC, but not in the sham control groups. This would suggest that the increase of GDNF in our study was not due to the potential injury-induced elevation of GDNF. We therefore hypothesized that GDNF produced by grafted BMSC that were preconditioned *in vitro* prior transplantation might provide a feedback mechanism after transplantation to maintain a high level of local GDNF, which creates niches that favors or facilitates the differentiation of new neuron or neuron-like cells from an undefined cell source (Supplemental Fig. S7). In addition, our observation that grafted BMSC stably maintained a neuronal phenotype for at least 28 days post-transplantation would suggest that BMSC have long-lasting effects on promoting neuron regeneration.

The contractility/tension of pyloric muscle was markedly improved 28 days post BAC-induced pylorus denervation. This functional restoration of muscular contractility strongly demonstrates the therapeutic potential of BMSC. While our study made a progress in elucidating the mechanism and the feasibility of BMSC-mediated therapeutic potentials, future studies are warranted. For example, the detailed characterization of the *in vivo*-regenerated PGP9.5/NSE/Tuj1/HuC/D/nNOS-positive neurons or neuronal structures should be performed to define their origin.

In summary, we demonstrate that *in vitro*-preconditioned BMSC promote *de novo* nerve regeneration and restore muscle contractility in pylorus-denervated rats through a GDNF positive feedback mechanism. Our study provides a novel concept for the mechanism responsible for BMSC-induced neuron regeneration and also a strong proof-of-principle for clinical application of BMSC-based therapy in ENS-related disorders. From clinical and practical standpoints, with the rapid technological advances in the area of endoscopy, BMSC can be endoscopically delivered anywhere in the GI tract, making it possible for future clinical application of segment-specific BMSC-based therapy with minimally invasive procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance Statement

Injury or neurodegenerative disorders of the enteric nervous system (ENS) cause gastrointestinal dysfunctions for which there is no effective therapy. We demonstrate that in vitro-preconditioned bone marrow mesenchymal stem cells (BMSC) promote de novo nerve regeneration and restore muscle contractility in pylorus-denervated rats. Our study provides a novel mechanism for BMSC-induced neuron regeneration and also a strong proof-of-principle for clinical application of BMSC-based therapy in ENS-related disorders. From clinical and practical standpoints, with new advances in endoscopy, BMSC can be endoscopically delivered anywhere in the GI tract, making it possible for future segment-specific BMSC-based therapy with minimally invasive procedures.

Figure 1. *In vitro* preconditioned BMSC exhibited a PGP9.5/Tuj1/HuC/D/nNOS-positive neuron-like phenotype

(A-D) BMSC at passage 6 were preconditioned by glial cell line-derived neurotrophic factor (GDNF) in fetal gut condition medium (FGCM). Cells were immunostained with neuronal marker (PGP9.5, NSE, Tuj1, HuC/D) and nNOS (colored panels). Images were obtained by fluorescent microscopy. Preconditioned cells expressed multiple neuronal markers [PGP9.5, NSE, Tuj1, HuC/D and nNOS]. Negative control (NC) in the presence of 2nd antibody (but absence of primary antibodies) showed no immunoreactivity. Nuclei were stained with DAPI (Blue). The far-right image of each colored panel is the overlay of the three images to its left. Representative of 5 independent experiments are shown. (E-I) The percentage of positivity of PGP9.5, NSE, Tuj1, HuC/D and nNOS in preconditioned BMSC were shown individually as bar graphs in the panel. Cells were counted in at least 20 fields (10X) from each independent experiment (n=5).

Figure 2. BAC (0.5%) effectively ablated ENS in pylorus which remained essentially neuron-free for at least 28 days

(A) A schematic illustration of region of interest (ROI) in rat ENS denervation model. BAC was applied to the 3-cm segment around the serosal surface of pylorus, with 1.5 cm each at proximal and distal side, respectively. The ROI in our study was defined as the 1-cm region right in the middle of the 3-cm BAC-treated. All *in vivo* experimental analyses were performed using this region (ROI). (B) Representative micrographs showing the immunoreactivity of neuron marker, PGP9.5, in 0.5% BAC treated group (right) and sham-operated group (left). No detectable PGP9.5 was observed in the BAC-treated tissues 28 days post-BAC treatment, in contrast to the sham operation controls. See more characterization at different doses of BAC in Supplemental Fig S3. (C) PGP9.5 levels in pylorus at day 28 examined by Western blot analysis with sham operation as control. Upper panel, representative Western blots; lower panel, statistical analysis of PGP9.5 expression (n=4-6). Data are shown as mean $\pm$ SEM. *P<0.01. IHC (upper panel) and IF (lower panel)

Figure 3. Allogeneically transplanted BMSC can survive in specific niches of pyloric wall and promote *de novo* regeneration of neuron-like cells/structures (which were not originated from grafted BMSC)

(A) Preconditioned BBM-labeled BMSC remained in the subserosal layer (where they were injected) one day after transplantation (Fig 3A, left panel, blue). Most of the transplanted BMSC migrated from serosal side to the longitudinal/circular muscle layer 7 days after transplantation (Fig 3A, middle panel, blue). [M, mucosal layer; SM, submucosal layer; LM, longitudinal muscle layer; CM, circular muscle layer; S, serosal layer]. (B) Transplanted BMSC promoted *de novo* regeneration of new neurons. At 28 day post-transplantation, most grafted BMSC migrated to the submucosal layer (SM), and survived, in the absence of any immunosuppressive agent or caspase inhibitor (Fig 3B-a, blue). Most of grafted BMSC maintained strong PGP9.5-positivity in SM (Fig 3B-d,f, SM, pink). A very small fraction of grafted BMSC were PGP9.5-negative and closely located in mucosa (Fig 3B-d,e, M, blue). A large quantity of regenerated PGP9.5-positive neurons was detected in predominantly muscle layer (and some in submucosa) (Fig 3B-b, red larger structures). Merged image show these regenerated neuron-like cells/structures (Fig 3B-d, red) had not originated from grafted BMSC because no blue color (BBM) was detected in any of these new neurons [see enlarged images (Fig 3B-e,f,g) of the areas marked with green rectangles (Fig 3B-d)].

Figure 4. The regenerated neurons induced by BMSC were positive for multiple neuron markers and mutually exclusive with grafted BMSC

(A) New neurons (in red, larger structures) exhibit strong expression of HuC/D (Fig 4A-a), Tuj1 (Fig 4A-b), and nNOS (Fig 4A-c), as compared to grafted BMSC [in blue or pink (overlay), smaller structures]. Representative from 15 rats are shown. (B) The regenerated PGP9.5-positive neuronal structures in the muscular layer are less ordered compared with that of normal control rats (sham controls with no BAC treatment). (C) Western blot analysis demonstrated dramatic increase of various neuronal markers (also shown in the immunohistology in (A) in pyloric wall of BMSC-grafted denervated rats. Quantitative analysis of Western blots (C, lower panel) by densitometry (normalized to β -actin graph), using one-tailed Student's *t*-test. Error bars denote SEM. $*P < 0.01$. Representative of at least 5 independent experiments are shown. Scale bar, 100 μ m.

Figure 5. Regenerated neurons and neuronal structures do not originate from grafted *in vitro* preconditioned GFP-BMSC

28 days post transplantation, BMSC from *in vitro*-preconditioned GFP-transgenic mice were detected in pyloric wall (GFP; green). The regenerated neuron-like structures (PGP9.5; red) did not colocalize with grafted GFP-BMSC. Representative images from the examination of 3 rats transplanted with GFP-BMSC, as well as 5 sham normal control rats. Scale bar, 100 μ m.

Figure 6. Both basal tension and EFS-induced relaxation of circular denervated muscle strips were significantly improved by BMSC transplantation

(A) Representative basal curves of isometric tension of circular pylorus muscle strips isolated from BAC-ablated group (lower panel), sham operation group (middle panel), and BMSC+BAC-treated group (upper panel). (B) The tension (analyzed as grams and normalized for the cross-sectional area of the strip (g/mm^2) of each treatment group is expressed as mean $\pm$ SEM under both basal. (C) Representative traces of neural response induced by electrical field stimulation of circular pylorus strips at increasing frequency of stimulation (1-8Hz) under NANC conditions ($5 \mu\text{M}$ atropine and $3 \mu\text{M}$ guanethidine) from BAC-ablated group, BMSC+BAC-treated group and sham operation group. (D) Quantification of circular pylorus strip relaxations in response to EFS under NANC conditions in BAC-ablated group, BAC-ablated followed by BMSC treatment group and sham operation group. One-way ANOVA showed significant differences between the BAC-ablated group and BMSC treatment group at all stimulation frequencies. * $P < 0.05$, **: $P < 0.01$. Results are mean $\pm$ SEM. of 8–11 strip preparations. (E) Quantitative analysis of L-NAME effect ($300 \mu\text{mol}/\text{L}$) on NANC-induced relaxations of pylorous muscle strips from the three experimental groups in response to EFS. Results are mean $\pm$ SEM. of 8–11 strip preparations.

Figure 7. GDNF positive feedback of BMSC maintains a high concentration of GDNF in BAC-treated pylorus

(A) GDNF, which was expressed at a very low level endogenously in BMSC, was dramatically enhanced by *in vitro* GDNF induction. The relative expression of GDNF RNA was examined by real time PCR. (B) 28 days after BMSC transplantation, a continuous, stable high level of GDNF expression was detected in deviated pyloric wall, comparing with sham injected group (upper panel). Relative GDNF level was normalized with β -actin (lower panel). * $P < 0.01$. Data was shown as mean $\pm$ SEM, and are representative of at least 3 independent experiments.