The Appendix as a Viable Source of Neural Progenitor Cells to Functionally Innervate Bioengineered Gastrointestinal Smooth Muscle Tissues

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ABSTRACT

Appendix-derived neural progenitor cells (NPCs) have both neurogenic and gliogenic potential, but use of these cells for enteric neural cell therapy has not been addressed. The objective of this study was to determine whether NPCs obtained from the appendix would differentiate into enteric neural subsets capable of inducing neurotransmitter-mediated smooth muscle cell (SMC) contraction and relaxation. NPCs were isolated from the appendix and small intestine (SI) of rabbits. Bioengineered internal anal sphincter constructs were developed using the same source of smooth muscle and innervated with NPCs derived from either the appendix or SI. Innervated constructs were assessed for neuronal differentiation markers through Western blots and immunohistochemistry, and functionality was assessed through force-generation studies. Expression of neural and glial differentiation markers was observed in constructs containing appendix- and SI-derived NPCs. The addition of acetylcholine to both appendix and SI constructs caused a robust contraction that was decreased by pre-treatment with the neural inhibitor tetrodotoxin (TTX). Electrical field stimulation caused relaxation of constructs that was completely abolished in the presence of TTX and significantly reduced on pre-treatment with nitric oxide synthase inhibitor (L-arginine methyl ester hydrochloride [L-NAME]). These data indicate that in the presence of identical soluble factors arising from intestinal SMCs, enteric NPCs derived from the appendix and SI differentiate in a similar manner and are capable of responding to physiological stimuli. This coculture paradigm could be used to explore the nature of the soluble factors derived from SMCs and NPCs in generating specific functional innervations.

INTRODUCTION

The enteric nervous system (ENS) is responsible for controlling digestion and propulsion of food along the gastrointestinal (GI) tract [1, 2]. Vast networks of neurons and glia with complex chemical coding are needed for the ENS to function properly [1]. Cells of the ENS are derived from the vagal and sacral levels of the neural crest and migrate during embryogenesis in a highly organized manner to fully colonize the fetal gut, including the appendix [3].

Neurodegenerative diseases affecting the GI tract are characterized by the disruption of the neuronal circuitry. These diseases result in motility disorders, loss of peristalsis, and impaired secretory functions.

Enteric neuropathic diseases can affect entire populations of neurons within certain regions of the gut or specific subpopulations of neurons, making these diseases extremely difficult to treat [4–6]. In the lower esophageal sphincter and the internal anal sphincter (IAS), achalasia is characterized by the inability of the sphincter to relax. This is often due to the absence of intrinsic inhibitory motor neurons. Other diseases such as diabetes and Parkinson’s disease also result in enteric neuropathies and lead to dysmotility. Current therapies are often unsatisfactory and do not replenish underlying factors...
neural deficiencies. Potential therapies include transplantation of neural stem cells to replace missing neuronal and glial cells. Regenerative medicine approaches seek viable sources of neural stem cells for use as cell therapies to treat enteric neuropathies and subsequently improve the function of the GI tract. The source of neural stem cells for cell therapies to treat ENS disorders remains a major challenge [7].

Embryonic stem cells, induced pluripotent stem cells, and autologous cells are strong candidates with therapeutic potential for neural regeneration. The use of autologous stem cells is considered ideal to avoid ethical concerns and to minimize tumorigenic potential; however, the challenge remains to identify the appropriate source for autologous cells. The ENS is a potential source of autologous neural progenitor cells (NPCs) [8]. Indeed, NPCs isolated from the myenteric plexus of rats have been shown to reaggregate in vitro [9]. Our previous studies have demonstrated that extensive neuroglial differentiation can be obtained in vitro using neuronal progenitor cells derived from adult rodent and human intestines [10–13]. Enteric NPCs isolated from the gut have been transplanted into the large intestine, where they migrated, differentiated into excitatory and inhibitory motor neurons, and formed ganglion-like structures [8]. Other studies demonstrated the presence of large amounts of easily accessible NPCs in the appendix [3, 14]. The appendix is an easily accessible autologous source of NPCs. Few studies in the literature have systematically characterized the differentiation and functionality of appendix-derived NPCs. We hypothesized that neural stem cells derived from the appendix would have the capacity to grow, differentiate, and function in bioengineered GI tissues similarly to neural stem cells derived from the small intestine (SI). The goal of this study was to compare the potential of the neural stem cells isolated from both the SI and the appendix to differentiate and innervate bioengineered GI constructs.

**MATERIALS AND METHODS**

**Reagents**

Media reagents were purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA, http://www.thermofisher.com) unless otherwise specified. Neural growth media consisted of neurobasal medium (Life Technologies; Thermo Fisher Scientific) containing N2 supplement (Life Technologies; Thermo Fisher Scientific), recombinant human epidermal growth factor (EGF 20 ng/ml; Stemgent, Cambridge, MA, https://www.stemgent.com), recombinant basic fibroblast growth factor (EGF 20 ng/ml; Stemgent, Cambridge, MA, https://www.stemgent.com), recombinant basic fibroblast growth factor (EGF 20 ng/ml; Stemgent), and antibiotics-antimycotics. Muscle media reagents were purchased from Invitrogen (Thermo Fisher Scientific) containing N2 supplement (Life Technologies; Thermo Fisher Scientific) supplemented with 2% FBS, B27 (Life Technologies; Thermo Fisher Scientific) supplement and antibiotic-antimycotics. Rat tail collagen (type I) was purchased from BD Biosciences (San Jose, CA, https://www.bdbiosciences.com), collagenase (type II) from Worthington Biochemicals (Lakewood, NJ, http://www.worthington-biochem.com), dispase and DNase from Roche Applied Science (Indianapolis, IN, https://lifesience.roche.com), and Hank’s Balanced Salt Solution (HBSS) from Thermo Scientific HyClone (Thermo Fisher Scientific).

**Isolation of Enteric Neuronal Progenitor Cells From the Small Intestine and Appendix**

All rabbit studies were performed with strict adherence to institutional animal care and use committee protocols approved by the Wake Forest Institute of Regenerative Medicine. SI and appendix tissues were obtained from New Zealand white rabbits (Charles River Laboratories International, Inc., Wilmington, MA, http://www.criver.com). Enteric NPCs were isolated from the small intestine, as described previously [10, 11, 13]. Briefly, SI tissues were emptied of their luminal content and washed extensively using HBSS. The tissues were minced and digested in a mixture of 0.8 mg/ml collagenase type II and 0.8 mg/ml dispase for 1 hour at 37°C with agitation. The supernatant was passed through a 70-μm cell strainer, and the pellet was subjected to another digestion, at the end of which the supernatant was also passed through a 70-μm cell strainer and combined with the initial digest. Cells were pelleted at 1,000g, washed, resuspended in neural growth media, and passed through a 40-μm mesh. Enteric neural progenitor cells were placed in nontissue culture-treated dishes and maintained at 37°C and 7% CO2. Appendix tissues were cleaned of their luminal content and washed extensively with HBSS. Tissues were minced and subjected to digestion in a collagenase-dispase mixture, as described. After centrifugation, cells were recovered by passing the pellet through a 70- and 40-μm cell strainers, resuspended in neural growth media, and cultured in nontissue culture-treated dishes at 37°C and 7% CO2.

**Culture of Sphincteric Smooth Muscle Cells**

Smooth muscle cells (SMCs) were isolated from IAS tissues of New Zealand white rabbits (Charles River Laboratories International, Inc.) following the method described previously [13, 15, 16]. Briefly, rabbit internal anal sphincters were isolated by sharp dissections and washed extensively in HBSS, followed by mincing. Minced tissues were digested with 1 mg/ml collagenase type II at 37°C for 1 hour with agitation. Digested tissues were pelleted at 600g for 5 minutes, washed with HBSS, and exposed to another digest, as before. Cells were collected by centrifugation and plated on tissue culture-treated dishes in muscle growth medium. Cells were cultured at 37°C and 5% CO2.

**Immunohistochemical Characterization of Isolated Cells**

NPCs obtained from the SI and the appendix were characterized by immunohistochemistry. Briefly, enteric neurospheres were fixed in formaldehdyde and blocked with 10% horse serum. Neurospheres were incubated with primary antibodies for p75 and Sox2 (1:200; Abcam, Cambridge, U.K., http://www.abcam.com) and nestin (1:200; AbD Serotec, Raleigh, NC, http://www.abdersotec.com) at room temperature. Appropriate fluorophore-conjugated secondary antibodies were then applied. Neurospheres were visualized using an inverted Nikon Ti-E fluorescence microscope (Nikon, Tokyo, Japan, http://www.nikon.com). Isolated sphincteric smooth muscle cells were stained using the same neuronal precursor markers and the neuronal marker βIII-tubulin and served as a control.

**Bioengineered Innervated Smooth Muscle Constructs**

Innervated smooth muscle constructs were bioengineered using either SI- or appendix-derived neural progenitor cells and IAS SMCs. Cells were used at 6 weeks after isolation. The method
of engineering in our laboratory was described previously [13]. Briefly, enteric neurospheres were recovered by centrifugation and dissociated into single cells using Accutase (Life Technologies). An estimated 200,000 enteric single NPCs were obtained after counting using a hemocytometer and then embedded in each collagen/laminin gel. Single cells were then pipetted onto a Sylgard-coated plate with a central cylindrical Sylgard post. After gelation, a second layer of collagen gel containing 500,000 IAS SMCs was pipetted on top of the neural layer. Neural differentiation media was added to the plate and incubated at 37°C to allow construct formation. At days 10–12 after formation, constructs were harvested for further evaluation.

**Immunohistochemistry of Bioengineered Constructs**

Constructs from both sources were fixed in 4% formaldehyde and embedded in paraffin. Cross-sections of 6-μm thickness were obtained, deparaffinized, and rehydrated. Sections were then blocked in 10% horse serum and incubated in primary antibody against neural markers: βIII-tubulin (1:150; Abcam), anti-choline acetyltransferase (anti-ChAT; 1:100; Abcam), and anti-neuronal nitric oxide synthase (anti-nNOS; BD Transduction Laboratories, BD Biosciences). Sections were stained for glial markers glial fibrillary acidic protein (GFAP; 1:200; Abcam) and S100b (1:100; Abcam). Slides were washed with 1× phosphate-buffered saline, and appropriate fluorophore-conjugated secondary antibody was applied. Sections were imaged using an inverted Nikon Ti-E fluorescence microscope.

**Western Blots of Bioengineered Constructs**

Proteins were extracted from bioengineered constructs with RIPA buffer and estimated spectrophotometrically using Bradford assays. Smooth muscle cells without coculture with neural progenitor cells were used as a control. Overall, 20 μg of protein from each sample was resolved electrophoretically and transferred to polyvinylidene difluoride membranes. Membranes were blotted with antibodies for neuronal βIII-tubulin, GFAP, nNOS, ChAT, and β-actin as a loading control. Corresponding horse-radish peroxidase-conjugated secondary antibodies and HyGLO chemiluminescence substrate (Denville Scientific Inc., South Plainfield, NJ, http://www.denvillescientific.com) were used to visualize bands with a Fujifilm imaging system (Fujifilm, Tokyo, Japan, http://www.fujifilm.com).

**Physiological Functionality Evaluation**

Physiological functionality of innervated smooth muscle constructs from both groups was evaluated using an isometric force transducer. Day 10–12 constructs were placed in an organ bath containing DMEM buffered with 25 mM HEPES. The tissue was connected to the measuring arm of the transducer and was maintained at 37°C throughout the experiment. A 15%–20% stretch was applied to the bioengineered tissues, and baseline was established for 30 minutes before treatment. Baseline was arbitrarily set to zero. The values are reported as changes in force (micro-Newton [μN]). Bioengineered tissues were treated with potassium chloride (KCl) and acetylcholine (Ach). Neural stimulation was achieved by electrical field stimulation (EFS) using parallel platinum-plate electrodes. EFS responses were evaluated in the absence and presence of neurotactin (tetrodotoxin [TTX]) and neuronal nitric oxide synthase inhibitor [Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME)].

**Statistical Analysis**

Force-generation data were acquired at 1,000 samples per second. Data were analyzed using GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, CA, http://www.graphpad.com). Second-order Savitzky-Golay smoothing was applied. One-way analysis of variance was used to compare means using GraphPad Prism. A *p* value <.05 was considered significant. Physiological studies were done on six bioengineered innervated smooth muscle constructs in each group. Values are expressed as mean ± SEM.

**RESULTS**

**Characterization of Isolated Cells**

Neural progenitor cells isolated from the small intestine and the appendix were allowed to grow in culture as single cells. At 1 week following their isolation, neural progenitor cells from both sources started forming small floating clusters, referred to as “neurospheres,” as shown in the bright-field images (Fig. 1A, 1C, 1E, 1G). Cells were maintained in culture 6 weeks and then were used for the purpose of the experiments listed in this study. Immunohistochemical (IHC) evaluation of the neural progenitor cells from both sources revealed positive stain for p75NTR (Fig. 1B, 1D). The neurospheres were also positive for Sox2 (Fig. 1F, 1H) and nestin (Fig. 1J, 1L). All of the antibody markers for neural crest-derived stem cells were shown to be reactive for rabbit, human, and mouse cells. The antigen for the p75NTR antibody was WM245 human melanoma cells. The antigens for Sox2 and nestin were recombinant human proteins corresponding to the C terminus and amino acids 1484–1500, respectively. The IHC results indicated that the neural progenitor cells obtained from both the SI and the appendix of the rabbits were derived from the neural crest. Isolated smooth muscle cells were allowed to grow for 6 weeks and then were used in the experiments. Isolated smooth muscle cells stained negative for the neuronal precursor markers and for neuronal marker βIII-tubulin, indicating no neuronal contamination in the smooth muscle culture.

**Immunohistochemical Characterization of Differentiation of SI- and Appendix-Derived Neural Progenitor Cells in Bioengineered Constructs**

Cross-sections of bioengineered constructs containing NPCs from the SI and the appendix were obtained and used for immunohistochemical studies. Both appendix and SI constructs stained positive for βIII-tubulin (Fig. 2A, 2F) and GFAP (Fig. 2B, 2G), indicating the presence of neuronal cells and glial cells, respectively. To further characterize different neuronal populations, constructs were stained for excitatory and inhibitory motor neuronal markers. Constructs stained positive for ChAT (Fig. 2C, 2H), indicating the presence of neurons that produce the major excitatory neurotransmitter in the gut, Ach. Both constructs also stained positive for the inhibitory neurotransmitter nNOS (Fig. 2D, 2I). Immunohistochemical studies demonstrated the presence of excitatory and inhibitory motor neurons in both types of constructs. Both constructs also stained positive for the glial marker S100b (Fig. 2E, 2J). These results indicate that neural progenitor cells derived from either the small intestine or the appendix have the potential to differentiate into mature neurons and glial cells.
Comparison of Levels of Differentiation of Neural Progenitor Cells Within Bioengineered Constructs

Western blot studies were performed to determine the relative expression levels of neuronal differentiation markers within bioengineered IAS constructs. Western blot analysis demonstrated that constructs containing SI- and appendix-derived NPCs expressed neuronal and glial markers βIII-tubulin and GFAP, respectively (Fig. 3A). No significant differences were observed in βIII-tubulin and GFAP expression, as assessed by densitometric analysis of band intensities. Staining of SMCs alone revealed no expression of neuronal or glial markers.

To further characterize and compare different neuronal populations, expressions of excitatory and inhibitory motor neuronal markers were assessed through Western blots (Fig. 3B). Constructs containing both SI- and appendix-derived NPCs demonstrated expression of nNOS and ChAT, indicating the presence of neurons that produce the major excitatory and inhibitory neurotransmitter in the gut. No significant differences in expression levels of nNOS or ChAT were observed between constructs regardless of origin of NPCs. Staining of SMCs alone revealed no expression of neural subset markers. These studies demonstrate similar levels of excitatory or inhibitory motor neurons in constructs containing SI- or appendix-derived NPCs, indicating similar differentiation patterns of NPCs isolated from different regions of the gut.

Physiological Functionality of the Bioengineered Constructs

Bioengineered innervated smooth muscle constructs using neural progenitor cells derived from either the small intestine or the appendix were compared for physiological functionality. Studies were conducted on constructs at days 10–12. Real-time force generation was performed using an organ bath connected to an isometric force transducer set-up. The smooth muscle constructs generated a spontaneous tone, a characteristic of tonic smooth muscle. All force values of contraction and relaxation are reported as Δ force. The quality of smooth muscle cells in both constructs...
To determine the cholinergic neuronal contribution, we pretreated the constructs with TTX (red trace). The same dose of Ach caused a contraction that was attenuated by 45%.

Relaxation was evaluated using electrical field stimulation. EFS parameters (5 Hz, 0.5 ms) were used to induce relaxation in the smooth muscle constructs. Constructs were placed between parallel platinum-plate electrodes. Both types of constructs relaxed in response to EFS (Fig. 5). Innervated smooth muscle constructs using SI NPCs relaxed to $-229.3 \pm 13.53 \, \mu N$ and constructs bioengineered using appendix NPCs relaxed to $-257.7 \pm 7.410 \, \mu N$. In both types of constructs, EFS-induced relaxation was completely abolished by TTX (red trace). These results indicated that the relaxation caused by EFS was purely neuronally mediated. In order to further characterize the different population of neurons present in the constructs, we used inhibitors for nNOS (L-NAME) and for nicotinic receptors (hexamethonium chloride) (data not shown). Both types of constructs were preincubated with L-NAME (green trace) for a period of 15 minutes, at the end of which EFS was applied. Inhibition of 40%–50% was observed in the constructs (Fig. 5). These results indicated that a functional population of nNOS neurons existed in the constructs. Pretreatment of the constructs with hexamethonium chloride abolished EFS-induced relaxation in both constructs. Inhibition caused by hexamethonium chloride indicated the presence of nicotinic receptors on the differentiated neurons.

**DISCUSSION**

The ENS is a vast neural network within the GI tract that is responsible for controlling the processes associated with digestion, including motility [17, 18]. To control these processes, the ENS has developed a great deal of complexity. All of the neurotransmitters found in the brain are also expressed by cells within the ENS, has numbers of neurons comparable to the spinal cord [1, 2, 18]. Enteric neuropathies are associated with decreased neural function and/or abundance within the gut. This often leads to diminished motility, and current treatments for diseases of the ENS are insufficient because they do not repair or reinstate a functional ENS within the gut. Cell therapies and tissue engineering provide curative methods of treating enteric neuropathies; however, the optimal cell source has not yet been identified.

The ENS resides in a continually changing environment in the gut that experiences constant insults, making plasticity and regeneration critical for proper functioning of enteric neurons [19, 20]. Well-established protocols have been developed to isolate enteric NPCs from the GI tract [21, 22]. When transplanted into rodents, these cells differentiated into neurons that re-established motility or function [23, 24]. In a recent study, enteric neural progenitor cells were compared with brain-derived progenitor cells [25]. Cells derived from the ENS had greater potential to migrate and differentiate than cells derived from the brain. Enteric NPCs isolated from the appendix have been gaining special attention recently because of their ability to proliferate and differentiate in culture. Because NPCs derived from the appendix and other parts of the gut arise from the neural crest [3], we anticipated similar behaviors of these cells. Human enteric neurospheres obtained from the appendix were confirmed to differentiate into neurons, more specifically, to dopaminergic fate [14]. The cells migrated and differentiated when transplanted into rat brain slices. In our study, we investigated the potential use of appendix-derived neural progenitor cells in innervating GI tissues. We previously established a technique for engineering...
GI tissues including IAS [13]. We were successful in innervating the engineered sphincters using enteric neural progenitor cells derived from the ganglionated plexi of the SI. The neural progenitor cells differentiated into functionally mature neurons, as demonstrated biochemically and physiologically. The engineered constructs maintained neuronal functionality following their implantation [26]. Using GI tissue engineering techniques in this study, we demonstrated that enteric NPCs isolated from the appendix had the ability to differentiate into functional neural subsets in a manner similar to NPCs isolated from the SI. These studies pave the way for the development of cell therapies using autologous enteric NPCs isolated from the patient’s own appendix as an additional potential method to establish ENS functions. Furthermore, these methodologies could be used to identify common factors that induce the differentiation of enteric NPCs.

This study suggests the appendix as an alternative source for neural progenitor cells that can be used to innervate engineered smooth muscle tissues. The neural progenitor cells derived from the appendix divide over an extended period of time and can remain in culture for a period > 6 months. The cells are typically passaged every month. Neurospheres start forming as early as 1 week after culture, and the neurosphere sizes vary from microscopic to macroscopic. Although the exact number of cells needed to treat enteric neuropathies remains to be determined, this study provided two potential sources from which neural progenitor cells can be obtained.

The appendix is an easy accessible alternative source of NPCs within the gut that can be isolated with minimally invasive surgical techniques from healthy and diseased patients [27]. Enteric NPCs can also be isolated from other regions of the gut including the SI. For the first time, in this study, the differentiation potential and functionality of enteric NPCs isolated from the appendix and SI were compared. The results indicated that NPCs from both sources had a similar abilities to differentiate into enteric neurons and glia within bioengineered IAS tissue constructs. This indicates that appendix-derived NPCs have the potential to differentiate into both neurons and glia. The differentiation of neural progenitor cells into excitatory (ChAT) and inhibitory (nNOS) motor neurons was demonstrated by immunohistochemistry and by Western blot. The presence of these neurons is essential for smooth muscle function.

Furthermore, NPCs isolated from both the appendix and the SI differentiated into mature functional excitatory motor neurons that expressed the major contractile neurotransmitter in the GI tract (ChAT). Partial inhibition of Ach contraction in the presence of the neurotoxin TTX showed that the response was partially mediated by the differentiated neurons. In addition, inhibitory motor neurons (nNOS) were identified. In the presence of L-NAME, relaxation was partially inhibited, which demonstrated the contribution of nNOS neurons to the relaxation response. Moreover, differentiated neurons were able to stimulate functional contraction and relaxation of SMCs within bioengineered constructs. These results suggest that appendix-derived NPCs can differentiate into functionally mature motor neurons, which are essential for reinstating motility. Taken together, these cells represent a valuable source for reinstating innervation in cases of neurodegenerative diseases of the gut.

GFAP- and S100b-positive cells seen in the constructs indicated that the neural progenitor cells derived from the appendix were capable of developing into support cells that play a major regulatory role in neuronal maintenance and integrity and in enteric neurotransmission [28]. S100b-positive cells were defined as late mature glial cells that lost their stem cell potential [29]. The ability of the
neural progenitor cells derived from the appendix to differentiate into glial cells is consistent with the results obtained using SI-derived NPCs and with our previously published results using SI NPCs to engineer GI tissues [10]. The differentiated neurons also demonstrated the capacity to synthesize and release functional neurotransmitters that induced the appropriate response of smooth muscle.

In order to treat complex and debilitating enteric neuropathies, cell therapy may be a potential cure. Identification of viable cell sources is paramount for the successful development of cell therapies. In this study, we identified the appendix, which is easily accessible, as a feasible source of enteric NPCs that can differentiate into neuronal subtypes in tissue microenvironments.

This study demonstrates that neural progenitor cells isolated from the appendix have the potential to differentiate into functional neurons in a manner similar to neural progenitor cells isolated from the ganglionic plexi of the GI tract. These results suggest that the appendix is a promising source for autologous neural stem cells required for cell therapy. These data indicated that in the presence of identical soluble factors arising from intestinal SMCs, enteric NPCs derived from the appendix and SI differentiated in a similar manner and were capable of responding to physiological stimuli. Future studies could explore the nature of the soluble factors derived from the SMCs and the NPCs in generating specific functional innervations.

CONCLUSION

This study demonstrates that neural progenitor cells isolated from the appendix have the potential to differentiate into functional neurons in a manner similar to neural progenitor cells isolated from the ganglionic plexi of the GI tract. These results suggest that the appendix is a promising source for autologous neural stem cells required for cell therapy. These data indicated that in the presence of identical soluble factors arising from intestinal SMCs, enteric NPCs derived from the appendix and SI differentiated in a similar manner and were capable of responding to physiological stimuli. Future studies could explore the nature of the soluble factors derived from the SMCs and the NPCs in generating specific functional innervations.

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AUTHOR CONTRIBUTIONS

E.Z. and S.L.R.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; S.R.: conception and design, collection and/or assembly of data; K.N.B.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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