Concise Review: Carbon Nanotechnology: Perspectives in Stem Cell Research

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Abstract
Carbon nanotechnology has developed rapidly during the last decade, and carbon allotropes, especially graphene and carbon nanotubes, have already found a wide variety of applications in industry, high-tech fields, biomedicine, and basic science. Electroconductive nanomaterials have attracted great attention from tissue engineers in the design of remotely controlled cell-substrate interfaces. Carbon nanoconstructs are also under extensive investigation by clinical scientists as potential agents in anticancer therapies. Despite the recent progress in human pluripotent stem cell research, only a few attempts to use carbon nanotechnology in the stem cell field have been reported. However, acquired experience with and knowledge of carbon nanomaterials may be efficiently used in the development of future personalized medicine and in tissue engineering. STEM CELLS TRANSLATIONAL MEDICINE 2013;2:376–383

Introduction
Nanotechnology is an emerging and rapidly developing field of modern science. This area of study entails the application of various industrial sectors, such as electronics, energy production, chemical engineering, and diverse fields of basic science and biomedicine. During the past decade, nanotechnology has been efficiently integrated into biomedical research, providing new methods for cell imaging, gene and small-molecule delivery, and scaffold design for tissue engineering purposes [1]. At present, it is possible to make “intelligent” nanodevices from proteins, lipids, synthetic molecules, and DNA [2–5]. A vast amount of research in the area of nanotechnology is performed and published online daily, and carbon derivatives are a small but attractive niche in this field.

Carbon-based nanomaterials can be beneficial tools for stem cell research involving multipotent and pluripotent stem cells, which are likely to play important roles in future regenerative medicine. Multipotent stem cells generally have limited differentiation potential toward cell lineages within the tissue of origin. Nonetheless, these cells are actively used in the clinic, for example, for the treatment of blood disorders (hematopoietic stem cells), and are under investigation in clinical trials for the treatment of spinal cord injury, musculoskeletal and other tissue repair, and tissue design (mesenchymal stem cells) (http://www.clinicaltrials.gov/) [6]. Pluripotent stem cells (embryonic and induced) can give rise to nearly all of the diverse cell types of the human body [7, 8]. These cells can be used for disease modeling and drug testing, and recent clinical trials of human embryonic stem cell (hESC)-derived retinal cells provided the first evidence of their suitability for cell therapies (http://www.clinicaltrials.gov/) [9, 10].

Although carbon allotropes have been extensively employed in industry and electronics and have been suggested for use in cancer treatment, their use in stem cell research and tissue engineering has only recently begun. In the adult organism, stem cells support tissue homeostasis and regenerate organs after injury [6]. Any malfunctions of regulatory pathways in the stem cell native microenvironment (niche) may cause disease or lead to the malignant transformation of cells [11, 12]. These processes have the potential to be controlled by nanotechnology approaches. Carbon nanodevices are able to regulate cellular behavior in vitro and in vivo at a single-cell level. Targeted molecule delivery offers a unique opportunity for reprogramming studies in vivo. Moreover, carbon-based nanomaterials can be used in scaffold design to recreate the specialized local microenvironment for optimal cell growth and differentiation to a specific cell type. Thus, this review highlights the latest trends in ongoing research; discusses the main applications of these intriguing carbon-based nanoscale tools, particularly graphene, carbon nanotubes, and their composites; and proposes future directions for carbon nanotechnology in the field of stem cell research.
CARBON ALLOTROPES

Carbon is an element involved in a number of natural processes on Earth. Carbon can form minerals and is an important constituent of the atmosphere. It is an indispensable component of chemical processes in living organisms and has routine applications in diverse, nonbiological areas of daily life. The numerous carbon forms (allotropes) identified to date include naturally occurring minerals (such as graphite, diamond, and coal) and fullerenes (such as buckyballs, graphene, and carbon nanotubes), which can be artificially synthesized and have more recently been found in nature [13–16]. The carbon atom has a valence of four, which determines the number of possible covalent bonds between carbon atoms within a molecule. Consequently, carbon allotropes differ according to the types of linkages (including the number of bonds between the atoms and the relative spatial orientations of the atoms) that form between the carbon atoms to create macromolecular structures (Table 1). These factors also determine the material characteristics of an allotrope.

For example, diamond has a typical three-dimensional crystal structure in which all four valences are used to create strong covalent bonds between atoms. This structure provides its superior mechanical and excellent optical properties (Table 1). Diamond has a high refractive index and transmittance, which made it a popular gemstone in fine jewelry manufacturing [13]. At present, diamonds can be artificially synthesized by various methods such as detonation, laser ablation or ion irradiation of graphite, high-pressure high-temperature technique, and chemical vapor deposition [17]. Nanodiamonds can be produced with a size range of 2–10 nm and can be chemically modified for the attachment of nucleic acids, proteins, or small molecules, thereby promoting research enabling biomedical applications. Diamond possesses intrinsic fluorescence, favoring its use in bioimaging in vitro and in vivo. The low reported toxicity of nanodiamonds stimulated their incorporation into tissue-engineered biodegradable polymer scaffolds to improve mechanical properties and has led to drug delivery studies [17].

Graphite is the most stable form of carbon under ambient conditions and has been used since ancient times in pottery painting and drawing [13, 18]. It has a planar, layered structure of stacked graphene sheets, which are weakly linked to each other by noncovalent van der Waals forces, allowing for the easy sliding of graphene layers.

Fullerenes: The main characteristic of fullerene family members is the covalent binding of carbon atoms to only three adjacent atoms in a planar structure. Thus, all valences are satisfied by two single bonds and one double bond.

Buckyball \((C_{60})\): This molecule is a remarkable icosahedral cage of 60 atoms symmetrically arranged into 12 pentagons and 20 hexagons. No two pentagons share an edge, and the molecule resembles a soccer ball. Higher molecular weight molecules, including ellipsoid forms, are also described.

Graphene: Graphene is a single layer of carbon atoms organized into a flat, two-dimensional, honeycomb (hexagonal) crystal lattice.

Carbon nanotube: This molecule can be described as a single graphene sheet rolled into a tube at a certain “chiral” angle. Carbon nanotubes can be single- (shown), double-, or multiwalled (one or a few tubes of different diameters inside each other).

“The term “fullerenes” is often used in regard to spherical fullerenes, such as the common “buckyballs.”

### Table 1. Carbon allotropes

| Allotropes    | Molecular structure |
|---------------|---------------------|
| Diamond       | ![Diamond Crystal](diamond-crystal.png) |
| Graphite      | ![Graphite Structure](graphite-structure.png) |
| Fullerenes    | ![Fullerene Structures](fullerene-structures.png) |
| Buckyball \((C_{60})\) | ![Buckyball](buckyball.png) |
| Graphene      | ![Graphene Lattice](graphene-lattice.png) |
| Carbon nanotube| ![Carbon Nanotube](carbon-nanotube.png) |

**Carbon Allotropes**

Carbon is an element involved in a number of natural processes on Earth. Carbon can form minerals and is an important constituent of the atmosphere. It is an indispensable component of chemical processes in living organisms and has routine applications in diverse, nonbiological areas of daily life. The numerous carbon forms (allotropes) identified to date include naturally occurring minerals (such as graphite, diamond, and coal) and fullerenes (such as buckyballs, graphene, and carbon nanotubes), which can be artificially synthesized and have more recently been found in nature [13–16]. The carbon atom has a valence of four, which determines the number of possible covalent bonds between carbon atoms within a molecule. Consequently, carbon allotropes differ according to the types of linkages (including the number of bonds between the atoms and the relative spatial orientations of the atoms) that form between the carbon atoms to create macromolecular structures (Table 1). These factors also determine the material characteristics of an allotrope.

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Graphite is the most stable form of carbon under ambient conditions and has been used since ancient times in pottery painting and drawing [13, 18]. It has a planar, layered structure of stacked graphene sheets, which we can see as a thin pencil line on a piece of paper (Table 1). Whereas graphite has been extensively used in industry (lubricants, batteries, the aerospace and automobile industries, etc.) [13], few reports have suggested biological applications, for instance, in surface chemistry modification studies [19]. In contrast, fullerenes, especially graphene and carbon nanotubes, have generated tremendous interest in many fields.

Buckminsterfullerenes (“buckyballs”), named after American engineer Richard Buckminster Fuller, first were produced by Nobel laureates Harold Kroto and colleagues in 1985 by the laser evaporation of graphite (Table 1) [http://www.nobelprize.org/nobel_prizes/chemistry/laureates/1996/] [20]. Only a few years later, spherical fullerenes were discovered in geological samples on Earth and in the cosmos (presolar environments) [15, 16]. As a fullerene
cage allows for the easy encapsulation of metal ions and, thus, eliminates their toxicity in vivo, buckyballs have attracted specific attention as excellent contrast agents for magnetic resonance imaging in clinical diagnostics. During exposure to focused visible light, fullerenes have the ability to generate reactive oxygen species (ROS), a property that can be used for photodynamic cancer therapy. Laser irradiation causes fullerenes to emit heat, which can be used for photothermal therapy. In addition, surface chemistry modification enables the binding of radioactive molecules, chemotherapeutic drugs, and targeting ligands, which facilitate the cell-specific delivery of the therapeutic payload [21].

Graphene is a flat monolayer of carbon atoms arranged in a two-dimensional hexagonal lattice (Table 1). Rolled graphene sheets represent carbon nanotubes (Table 1) [14]. Because of their unique molecular structure, both graphene and carbon nanotubes possess extraordinary electrical, thermal, and physical properties. These materials are widely used in industry and biomedical fields and are discussed in detail below.

**GRAPHENE IN INDUSTRY AND BIOMEDICINE**

Graphene began to attract much attention after comprehensive studies performed in 2004 by Novoselov et al. [22]. Later, in 2010, Konstantin Novoselov and his colleague Andre Geim were awarded the Nobel Prize in Physics (http://nobelprize.org/nobel_prizes/physics/laurcates/2010/). Scientists were able to prepare graphene sheets as small as one atom thick by mechanical exfoliation of graphite and characterized its electronic properties of such carriers can be employed to track gene or drug delivery and to apply thermal therapy to a restricted body site [38, 39].

Further studies by Park et al. have demonstrated the preference of multi-potential stem cells (iPSCs) revealed that glass coverslips coated with oxidized graphene enhanced cell attachment, proliferation, and differentiation versus unmodified graphene and glass. Unfortunately, the authors did not provide a direct comparison with traditional tissue culture-treated plastic, which is also oxidized (hydrophilic) and therefore improves cell adhesion and growth compared with untreated hydrophobic polystyrene [52, 53].

Further studies by Park et al. have demonstrated the preferential differentiation of human neural stem cells (an immortalized cell line) to neurons on laminin/graphene-coated glass versus cells on laminin/glass. Researchers have also showed the
possibility of electrical stimulation of differentiated cells on graphene substrates [54]. Electroconductive materials have been proposed to be especially beneficial in the generation of neural and cardiac tissue by improving cell growth and electrophysiological characteristics [55–57]. Specific interest is currently devoted to the fabrication of neural interfaces, which may be used as implantable devices for the restoration of central nervous system functions [55, 58]. In a recent report, Zhou et al. were able to produce 2D and 3D electrospray scaffolds via layer-by-layer deposition of graphene-heparin/poly-L-lysine polyelectrolytes. These electroactive scaffolds supported mouse cortical neuron attachment and neurite outgrowth [59].

Thus, graphene can be an effective tool in the design of microenvironments for stem cell growth and the control of cellular behavior and fate. Graphene would be especially useful in the surface chemical modification of cell culture substrates, allowing for the attachment of multiple bioactive molecules of choice. In addition, as published studies have shown, cardiac and neural tissue engineering would greatly benefit from the electrical properties of this nanomaterial.

**APPLICATIONS OF CARBON NANOTUBES**

Similar to graphene, carbon nanotubes (CNTs) have been extensively used in different sectors of industry, biomedicine, and science. Known as early as 1952 [60], CNTs became a subject for intensive research in 1991 after a publication in the journal Nature by Iijima, which highlighted the unique properties of CNTs [61]. This paper showed that graphitic carbon could be synthesized in the form of single-atom-thick, nanometer-diameter and micrometer-length cylinders. To date, CNTs can be produced as single-, double- [62], and multiwalled molecules with closed or open tips and demonstrate chirality-dependent electronic properties (metallic or semiconducting) [63].

The electrical and mechanical properties of CNTs give them a wide range of applications in electronics, such as transparent electrodes, nanowires, supercapacitors, transistors, and switching mechanisms. CNTs can be used for catalytic reactions in fuel cells and as highly responsive sensors for a wide range of molecules in the chemical industry and medicine [64, 65]. By incorporating CNTs into cotton fabrics, it is possible to manufacture protective clothing with fire-resistant, water-repellent, and improved mechanical characteristics [66]. CNTs are an interesting material for the design of “intelligent textiles” for healthcare, sports, infotainment, fashion, security, and other applications [30]. Interestingly, CNTs exhibit distinct diameter-dependent colors, a feature that will likely be used in the production of colored conductive coatings and paints [67, 68]. A number of biomedical applications of CNTs are proposed and outlined in [69]. These applications are not limited to bioimaging and diagnostic usage; studies have focused on cell-CNT interactions and immune response, and most promisingly, CNTs have been reviewed as a tool for cancer therapies [69].

The cellular uptake of CNTs is still not fully understood, and mechanisms such as passive diffusion and endocytosis have been proposed [70–72]. Cellular uptake may depend on cell type and CNT properties such as size, type, and functionalization—covalent and noncovalent linking of chemical groups and different molecules on a CNT backbone or the filling of the internal cavity of the nanotube [69, 70, 73]. In 2007, Kostarelos et al. reported efficient single- and multiwalled CNT uptake by different mammalian cell types and prokaryotic cells. CNTs were observed in the perinuclear region of human epithelial cells (A549 cell line) after only 2 hours of incubation [74]. The authors did not establish the specific mechanism of CNT uptake but showed that uptake was not dependent on the use of a specific type of CNT functionalization [74]. Interestingly, Villa et al. observed that human dendritic cells were able to internalize single-walled CNTs by macropinocytosis within a few minutes of incubation [71].

A few research groups have carried out computational simulations of single-walled CNT uptake, proposing a “nanoneedle”-like mechanism of passive diffusion through the membrane bilayer [72]. It was shown that CNT functionalization with ammonium groups does not significantly affect the process. However, open-ended CNTs could damage the phospholipid bilayer and induce local rearrangements of the cell membrane [72]. Such an easy method for molecule delivery into a cell is a very attractive tool for cell biologists and cancer researchers working with homogeneous cell populations or established cell lines. However, the nonspecific nature of CNT uptake makes targeted drug or gene delivery problematic. Thus, researchers have begun to develop methods for limiting CNT uptake to only cells of interest, using CNT shape modifications and attaching cell-specific antibodies to initiate receptor-mediated endocytosis [75, 76].

A number of methods for the chemical modification of CNTs have been developed, facilitating their diverse applications as carriers of different functional groups and molecules [77]. The cellular uptake of exogenous particles can also depend on the cell membrane and particle biophysical characteristics, such as charge, hydrophobicity, particle size, roughness, functional groups, and ligands [4], which should be considered when designing nanodevices. Recent literature includes extensive ongoing studies involving CNTs with the intention to treat cancer by the target-specific delivery of drugs, nucleic acids, bioactive proteins, and small molecules to kill tumor cells or to control abnormal gene or protein expression [73, 78]. In addition, Benincasa et al. proposed CNTs as an efficient antifungal drug delivery agent [79].

CNTs possess a few important physical characteristics that can be applied for cell tracking and the targeted killing of cancer cells. CNTs demonstrate Raman scattering (a laser-generated inelastic scattering of light), which permits their structural characterization and continuous tracking in vivo in animals [80, 81]. Under near-infrared laser light, CNTs convert radiation into heat, a feature that can be used for cancer cell- and tissue-specific photothermal ablation [76, 82].

Uncertainties regarding CNT toxicity and clearance from the host body still exist. Fortunately, recent studies demonstrate that CNT toxicity depends on multiple factors such as the purity of CNTs and their type, length, and surface chemistry, among other factors [70]. Cytotoxicity may differ in vitro and in vivo, may depend on whether CNTs are dispersed or immobilized in ECM [70], and may vary for cells grown in a monolayer or in aggregates [83]. Pietroistui et al. reported embryonic toxicity of single-walled CNTs in a mouse model [84]. However, current methods of CNT functionalization reveal minimum side effects, and CNTs were shown to be efficiently removed from the mouse body through the biliary and renal pathways [85, 86]. Strikingly, recent studies have revealed the biodegradation of functionalized CNTs in situ by oxidative enzymes such as horseradish peroxidase and phagolysosomal simulating fluid in the presence of hydrogen peroxide, in vitro inside myeloid cells and in vivo in the...
mouse brain cortex [87–89]. However, the toxicity and fate of CNT degradation products still need to be evaluated.

In recent decades, CNTs have been actively studied for possible applications in tissue engineering. Single-walled and multi-walled CNTs have been used as thin 2D films with or without ECM proteins and have been combined with biodegradable polymers such as poly(lactic-co-glycolic acid) (PLGA) or poly(l- lactic acid) to improve the hardness of 3D scaffolds and increase cell attachment by modifying surface roughness [70]. Most experiments so far have been directed toward the development of methods for bone and neural tissue regeneration.

CNT-based composites favor mesenchymal stem cell attachment and osteogenic differentiation [90, 91] and facilitate bone repair in rats [92]. Hydroxyapatite and chitosan scaffolds incorporating CNTs are another option for efficient bone formation [93]. Shin et al. developed a 3D CNT-hydrogel platform for tissue engineering studies, which has tunable mechanical properties and enables substrate surface patterning [94]. Others have suggested applications of single-walled CNT composites as antimicrobial agents in biomedical implants [95].

The electroconductivity of CNTs can be effectively used for cardiac and neural tissue repair. Thus, the electrical stimulation of rat cardiomyocytes deposited on CNT-based scaffolds improved their electrophysiological characteristics [96] while inducing the cardiac differentiation of hMSCs [57]. More excitingly, it has been reported that CNT scaffolds enhanced the neural differentiation of hMSCs [97] and, in animal models, improved neural cell branching and the synaptic activity of cells in a monolayer culture as well as in tissue explants [55, 98, 99]. Direct CNT-neuron contacts favor the back-propagation of action potentials, contribute to synaptic dynamics and improve network connectivity in substrate-cell interfaces. In the future, these features may have applications in implantable hybrid cell-nanomaterial devices, possibly allowing for information processing and control over or rescue from conditions caused by certain central nervous system disorders [55].

To date, few attempts have been made to study human pluripotent stem cell (hPSC) behavior on CNT-based scaffolds, particularly for neural differentiation. One group reported a high yield of Nestin-positive cells from spontaneously differentiated hESCs on collagen I/single-walled CNT composites versus cells differentiated on gelatin or collagen substrates alone [100]. Poly(methacrylic acid)- and poly(acrylic acid)-grafted multiwalled CNT films or silk fibroin/CNT composites promoted the enhanced outgrowth of Nestin and βIII-tubulin-positive cells from hESC-derived embryoid bodies compared with traditional poly-l-ornithine-coated glass coverslips [101, 102]. Although tissue-engineering applications of hPSCs are still in their infancy, carbon nanotechnology offers tremendous perspectives for stem cell research.

**Perspectives of Carbon Nanomaterials in hPSC Research**

hPSCs have attracted much attention from scientists and physicians because of their nearly unlimited proliferative potential and capability to differentiate into almost any type of cell in the human body [7, 8]. Recently, hESC-derived retinal pigment epithelium cells were successfully used to treat age-related macular degeneration and Stargardt’s macular dystrophy in clinical phase I/II trials [10]. As stem cell research moves rapidly toward personalized medicine, histocompatible hPSCs can be generated by reprogramming patient-specific somatic cells such as fibroblasts or blood cells to a pluripotent state—a discovery that led to the Nobel Prize in Medicine in 2012 (http://www.nobelprize.org/nobel_prizes/medicine/laureates/2012/) [8]. At present, a number of disease-specific human iPSC lines have been generated, providing the opportunity for disease modeling and the development of progressive therapies [9]. Another rapidly developing area of stem cell research is the direct reprogramming of somatic cells to tissue-specific cells [103]. This strategy provides a shortcut that eliminates the need to establish human iPSC lines and then differentiate them into the desired cell types. However, there are still a few obstacles preventing wide clinical applications of hPSCs [104]:

- There is no safe and efficient method to generate patient-specific hPSCs or tissue-specific cell types. Most studies have employed viruses, although a few reports have used episomal vectors, RNAs, or active proteins for cell reprogramming [105].
- Differentiation methods still need to be improved to generate functional cell types that will be able to adapt and function in the host environment.
- Reporter cell lines are needed to trace or define tissue-specific progenitor cells and efficiently eliminate undifferentiated hPSCs from tissue-specific progenitors to avoid tumor formation after cell transplantation to the patient.
- The genetic correction of disease-inducing mutations in vitro requires multiple manipulations of cells in long-term cell culture. However, any manipulations of cells outside the body, even their short-term expansion in vitro, can cause phenotypic and genotypic changes.

Carbon-based nanomaterials can be an efficient solution to the problems listed above. The ability to attach various molecules, including cell-specific ligands, to carbon molecules allows for the targeted transport of the payload (such as transcription factors, oligonucleotides, small RNAs, synthetic molecules, or chemicals) to regulate gene expression, make a cell traceable (by introducing a fluorescent probe) or simply to deliver a drug to eliminate a cell [73, 76, 106]. Carbon nanoconstructs can operate at the single-cell level in a highly specific manner and hold great promise as a future tool for stem cell research and regenerative medicine.

Because of their easy cellular uptake, carbon nanomolecules can be used for the generation of reprogrammed cell types as a virus-free alternative to existing methods [71, 72, 74]. The reprogramming of human somatic cells to iPSCs with single-walled CNTs was attempted a few years ago, but the results were not published [107, 108]. To date, with a growing knowledge of carbon derivatives, nanomolecules can be used in a directed cell differentiation in vitro studies. Further, CNTs can be used for cell tracking in in vivo experiments, and may even be an efficient tool in eliminating potentially tumorigenic hPSCs that did not complete their differentiation into a functional cell type. Moreover, after experiments “in a dish,” scientists have already demonstrated in a mouse model that somatic cells can be reprogrammed to a desired cell type in vivo [109, 110]. Recent publications by Garriga-Canut et al., describing the elimination of Huntington’s disease symptoms in model mice with adeno-associated virus-delivered zinc finger proteins [111], and by the Glazer group, describing gene editing in human hematopoietic cells using polymer PLGA nanoparticles encapsulating peptide nucleic acids and DNA molecules [112], show the possibility of restoring gene function and correcting genetic diseases in vivo. Similar to findings of published studies, carbon nanovehicles can be used...
for the genetic correction of mutated genes, thus eliminating the negative effects of manipulations outside the cell niche. The importance of the development of in vivo target-specific therapeutic strategies becomes even more pronounced with the growing awareness about somatic cell mosaicism in humans [113, 114]. Carbon nanodevices have the potential to change current strategies in stem cell research and medicine. In the future, it will likely be possible to stimulate endogenous stem cell pools, perform reprogramming of terminally differentiated cells or eliminate undesired cell types undergoing transformation in vivo in the patient’s body.

Carbon nanomaterials offer great opportunities for the design of 3D microenvironments for hPSC differentiation and tissue engineering. Their stable and simple chemical structures, electrical conductivity, high surface area versus nanoscale size, and capability of micropatterning make carbon derivatives a multitasking material for scaffold design. The attachment of ECM molecules of interest and control over stiffness, roughness, and cell substrate topography allow researchers to mimic numerous factors of the native tissue-specific cellular microenvironment [70]. These materials may facilitate a better understanding of hPSC biology, the development of novel differentiation systems, and the design of complex tissues, such as cardiac, neural, and bone. Thus, hPSC research involving carbon nanotechnology provides a perfect base for the development of new anticancer strategies and cell therapies and promises novel avenues for tissue and organ regeneration (Fig. 1).

CONCLUSION

Nanotechnologies effectively integrate into all areas of modern human life. The latest methods of diagnostics and therapies will soon provide accurate and personalized medical care. The industrial success of carbon nanomaterials and the recent progress of carbon derivatives in tissue engineering and anticancer studies provide great opportunities for stem cell researchers. Multipotent stem cells and hPSCs have already proven their usefulness in basic science, disease modeling, drug testing, and regenerative medicine. The introduction of advanced carbon nanotechnology to stem cell research will stimulate new directions and developments in science and medicine as a whole.

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AUTHOR CONTRIBUTIONS

M.V.P.: manuscript writing, editing and preparing figures.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The author indicates no potential conflicts of interest.

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