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Accelerating bioelectric functional development of neural stem cells by graphene coupling: Implications for neural interfacing with conductive materials

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ABSTRACT

In order to govern cell-specific behaviors in tissue engineering for neural repair and regeneration, a better understanding of material-cell interactions, especially the bioelectric functions, is extremely important. Graphene has been reported to be a potential candidate for use as a scaffold and neural interfacing material. However, the bioelectric evolvement of cell membranes on these conductive graphene substrates remains largely uninvestigated. In this study, we used a neural stem cell (NSC) model to explore the possible changes in membrane bioelectric properties – including resting membrane potentials and action potentials – and cell behaviors on graphene films under both proliferation and differentiation conditions. We used a combination of single-cell electrophysiological recordings and traditional cell biology techniques. Graphene did not affect the basic membrane electrical parameters (capacitance and input resistance), but resting membrane potentials of cells on graphene substrates were more strongly negative under both proliferation and differentiation conditions. Also, NSCs and their progeny on graphene substrates exhibited increased firing of action potentials during development compared to controls. However, graphene only slightly affected the electric characterizations of mature NSC progeny. The modulation of passive and active bioelectric properties on the graphene substrate was accompanied by enhanced NSC differentiation. Furthermore, spine density, synapse proteins expressions and synaptic activity were all increased in graphene group. Modeling of the electric field on conductive graphene substrates suggests that the electric field produced by the electronegative cell membrane is much higher on graphene substrates than that on control, and this might explain the observed changes of bioelectric development by graphene coupling. Our results indicate that graphene is able to accelerate NSC maturation during development, especially with regard to bioelectric evolvement. Our findings provide a fundamental understanding of the role of conductive materials in tuning the membrane bioelectric properties in a graphene model and pave the way for future studies on the development of methods and materials for manipulating membrane properties in a controllable way for NSC-based therapies.

1. Introduction

It is critical to develop new materials for manipulating neural stem cell (NSC) behavior for neural regeneration and tissue engineering. Current strategies mostly focus on the different

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biochemical modifications of the materials, while less effort had been made to explore how the physical properties of the materials affect NSC behaviors. The clearest effect of the materials on cells is their ability to influence membrane structure or function. It is well known that ion channels and pumps in the membrane play critical roles in cell function, including proliferation, migration, and apoptosis, in a wide range of cells [1]. In particular, the current literature has described the regulatory role of bioelectric membrane gradients and signaling in a variety of tissues during development, maturation, and regeneration [2], suggesting the importance of being able to manipulate these bioelectric membrane properties in regenerative medicine.

Graphene, a 2-dimensional monolayer of carbon atoms, has been at the forefront of nanotechnology due to the material's unique electrical, mechanical, and thermal features. It has recently been considered to be a promising candidate for the fabrication of ultrafast nanoelectronic devices, transparent electrodes, nanocomposite materials, and biomedical materials [3]. It has already been utilized in a variety of biomedical applications, including cellular imaging and drug delivery [4], bio-analysis [5], stem cell research [6,7], and even photothermal therapy for treating tumors [8]. Recently, we and other groups discovered the possibility of using graphene as a neural interfacing material because it could promote neurite sprouting and outgrowth in human neuroblastoma (SH-SY5Y) cell culture [9], PC-12 cells [10], primary cultures of hippocampal neurons [11], and direct NSC differentiation to neurons [12,13], facilitate NSCs differentiation into neurons on graphene nanomesh semiconductors and form neuronal fibers [14,15]. In addition, more and more studies have shown that graphene exhibits the potential capability of manipulating the fate of stem cells. For instance, graphene-based materials are capable of inducing NSC differentiation into the neuronal lineage [7,16], controlling and even accelerating the differentiation of mesenchymal stem cells [6,17–22], and regulating the behaviors of other types of stem cells, including pluripotent stem cells and embryonic stem cells [23–25]. These pioneering studies clearly demonstrate the great potential of graphene-based materials in cell therapies. However, the underlying mechanisms behind the altered cell behaviors, such as enhanced differentiation and promoted cell growth, remain largely unknown.

The strong connections between cell functions and the cell membrane's bioelectric properties inspired us to investigate whether graphene can regulate NSC development and the maturation of their progeny by affecting the bioelectric properties of the cell. In this work, we studied the impact of graphene on the maturation of the electrophysiological state during NSC development, including the passive and the active bioelectric properties and subsequent choice of NSC fate.

2. Materials and methods

2.1. Graphene film preparation

Graphene samples were synthesized according to the previously published CVD method [26]. Briefly, a thin copper foil (5 cm × 5 cm) was heated to 1000 °C and annealed for 20 min under H_2 and Ar gases, followed by exposure to H_2 and CH_4 for 5 min. The films were then cooled down from 1000 °C to room temperature under H_2 and Ar gases. Graphene films were removed from the copper foils by etching in an aqueous solution of iron nitrate. After the copper film was dissolved, a TCPS substrate was brought into contact with the graphene film and it was pulled from the solution to produce the graphene/TCPS substrate. The graphene/TCPS substrates were mounted with a chamber and immersed in milli-Q water overnight to remove any residual soluble toxic components. After sterilization with 75% alcohol, the graphene/TCPS substrates were successively soaked into sterilized PBS buffer and coated with laminin solution (20 mg/ml, 37 °C overnight, Sigma, USA) in PBS. Prior to cell seeding, the graphene films were soaked in proliferation medium overnight.

2.2. Characterization of graphene substrates

The transmittance of graphene was measured with a UV/Vis spectrometer (LAMBDA 25, PerkinElmer, Singapore). Glass microscope slides were cut into rectangles of 0.9 cm × 2.6 cm to fit into the sample holder. A blank glass slide was used as a reference for each measurement. The crystallinity and the number of layers present within the graphene were examined by Raman spectrometry (lamRAM HR800, HORIBA, France) and TEM (Tecnai G2 F20 S-Twin, FEI, USA). The surface morphologies of the graphene and TCPS were determined by AFM (Dimension 3100, Veeco, USA) using tapping mode operated at room temperature. The surface chemistry of the graphene film was examined by XPS (Axis Ultra DLD, Kratos, UK) with an Al Ka X-ray source operated at 40 eV. The morphology of the graphene films was examined by the scanning electron microscope (SEM) (Quanta 400 FEG, FEI, USA).

2.3. NSC culture under proliferation and differentiation conditions

NSCs were derived from both hemispheres of the hippocampus of postnatal day 1 ICR rats (Animal Center of SooChow University). The hippocampus was separated from blood vessels and meninges and collected in a Falcon tube in Hank's balanced salt solution (HBSS) at 4 °C, then rinsed with HBSS two times. After centrifugation (1000 rpm for 5 min), tissues were digested in TryplE (Life Technologies, USA) for 15 min at 37 °C then gently triturated mechanically by using pipet tips. NSCs were suspended in DMEM-F12 medium containing 2% B-27 and cultured in a humidified atmosphere with 5% CO_2 at 37 °C. The passage of NSCs was carried out every 7 days during culturing. For proliferation studies, NSCs were seeded at a concentration of 5×10^4 cells/mL in proliferation medium consisting of DMEM-F12 medium with 2% B-27 supplemented with 20 ng/mL EGF and 20 ng/mL FGF-2 (R&D Systems, USA). For differentiation studies, NSCs were seeded at a similar concentration in DMEM-F12 medium containing 2% B-27 supplemented with fetal bovine serum (GIBCO, USA) and 1 μM retinoic acid (Sigma). The care and use of animals in these experiments followed the guidelines and protocol approved by the Care and Use of Animals Committee of Southeast University. All efforts were made to minimize the number of animals used and their suffering.

2.4. Immunofluorescence

Cells were washed with PBS, fixed in 4% paraformaldehyde for 45 min, blocked in PBS containing 2% BSA, and permeabilized with 0.1% triton X-100 for 90 min. The cells were incubated with primary antibodies for 90 min and then incubated with secondary antibodies for 60 min followed by DAPI staining. The antibody panel included primary antibodies against GFAP, Ki67 (Abcam, USA), Tuj-1, O4, tubulin, vinculin (Sigma), and TREK-1 (Santa Cruz Biotechnology, USA). For imaging dendritic spines, cells have been transduced with lentivirus encoding mCherry-actin. pLVX-mCherry-actin vector (Clontech, USA) was used for packaging the lentivirus. The cells were imaged at DIV 21.

2.5. WST-based cell proliferation assay

The WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt]