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Electrically driven intracellular and extracellular nanomanipulators evoke neurogenic/cardiomyogenic differentiation in human mesenchymal stem cells

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ABSTRACT

Nanomechanical intervention through electroactuation is an effective strategy to guide stem cell differentiation for tissue engineering and regenerative medicine. In the present study, we elucidate that physical forces exerted by electroactuated gold nanoparticles (GNPs) have a strong influence in regulating the lineage commitment of human mesenchymal stem cells (hMSCs). A novel platform that combines intracellular and extracellular GNPs as nano-manipulators was designed to trigger neurogenic/cardiomyogenic differentiation in hMSCs, in electric field stimulated culture condition. In order to mimic the native microenvironment of nerve and cardiac tissues, hMSCs were treated with physiologically relevant direct current electric field (DC EF) or pulsed electric field (PEF) stimuli, respectively. When exposed to regular intermittent cycles of DC EF stimuli, majority of the GNP actuated hMSCs acquired longer filopodial extensions with multiple branch-points possessing neural-like architecture. Such morphological changes were consistent with higher mRNA expression level for neural-specific markers. On the other hand, PEF elicited cardiomyogenic differentiation, which is commensurate with the tube-like morphological alterations along with the upregulation of cardiac specific markers. The observed effect was significantly promoted even by intracellular actuation and was found to be substrate independent. Further, we have substantiated the participation of oxidative signaling, G0/G1 cell cycle arrest and intracellular calcium $[Ca^{2+}]_i$ elevation as the key upstream regulators dictating GNP assisted hMSC differentiation. Thus, by adopting dual stimulation protocols, we could successfully divert the DC EF exposed cells to differentiate predominantly into neural-like cells and PEF treated cells into cardiomyogenic-like cells, via nanoactuation of GNPs. Such a novel multifaceted approach can be exploited to combat tissue loss following brain injury or heart failure.

1. Introduction

Directed differentiation of stem cells towards different lineages has been a subject of extensive research interest in last few decades. Although early approaches using biochemical cues such as growth factors and cytokines to regulate stem cell differentiation are well accepted, recent experimental results demonstrate the pivotal role of adhesive and physical signals in explicitly regulating stem cell response [1,2]. Therefore, recreating this native environment using synthetic matrix with multiple cell-instructive cues for guiding stem cell differentiation has become the prime focus for both basic biological studies and regenerative medicine [2]. Unfortunately, an incomplete understanding of the critical mechanical and biochemical features limits the design of artificial culture platforms capable of regulating stem cell fate [3,4].

Lately, a number of studies have shown that stem cells can sense and transduce external physical cues such as shear stress, mechanical strain, matrix nanotopography, chemistry and surface stiffness [2]. Among the seminal observations made so far, more attractive is the concept of nanoscale topographical manipulation, as the ECM *in vivo* features topography in the nanoscale regime [5]. Nanotopographic stimuli are known to create multiple signal initiation points, presenting a bi-directional communication route that is translated through adhesions, intracellular tension and mechanotransduction [6,7]. For instance, MSCs grown on grating-

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like topographies acquired elongated morphology that triggered a significant up-regulation of neuronal markers, suggesting the induction of neural differentiation [5].

Apart from surface topography, electrical cues are also being exploited as powerful tool for tissue engineering applications. The concepts of bioelectricity and the existence of DC gradient of voltages (endogenous electrical fields) in developing and regenerating tissues were established decades ago [8]. Significantly, its biological role and therapeutic potential may be far more cardinal than expected as it overrides other physical cues in guiding cell migration and orientation of cell division [9]. The fact that nerve, muscles and glandular cells all make use of endogenously generated EF to conduct impulses is known for years [10]. It is unquestioned that endogenous currents are involved in major biological processes such as embryogenesis, wound healing, repair, remodeling, and perhaps growth, rather than being just an epiphenomenon. In this framework, the application of external EF to cells has a long and combative history. An elegant demonstration by Hinkle et al. showed that a small applied electric field can both direct the growth of amphibian neurites towards negative pole *in vitro* and also determine the orientation of the bipolar axis assumed by muscle cells developing *in vitro* from spherical myoblasts [11]. In the clinic, EF stimulation is extensively used in several settings, in order to activate the damaged or disabled neuromuscular system, (especially PNS, CNS and spinal cord injury) as well as to treat musculoskeletal disorders (such as bone healing, ligament healing, articular cartilage repair) and to delay the progression of osteoarthritis [12]. Additionally, external EF therapy is considered as a viable strategy for pain control, disability, muscle spasm, and improved physical function [13]. Such a physiological relevance attracted numerous investigators to study the effects of applied electric field on cellular functionality in culture, with the motive to mimic the *in vivo* situation [14–18]. In addition to their central role in NSC differentiation and neurite outgrowth *in vitro* [11,19–21], EF stimulation is reported to drive adipose/bone marrow derived stem cells as well as fibroblasts to differentiate into cardiac-type [22–24].

In this regard, biomimetic design strategies incorporating interactive effects of EF stimulation and conducting substrates have gained wider acceptance in tissue engineering. Such an approach can recapitulate the electrical environment of electroactive cells (e.g., cardiac or neural cells) by helping them to regulate cell–cell and cell-matrix communications [25–27]. Despite a long history of toxicity issues [28], carbon nanostructures (graphene, CNT etc.) incorporated substrates were employed as conventional cell-adhesion layer for promoting differentiation of electrically excitable cells due to its intrinsic conductivity and nanoscale features [29,30]. On the other hand, the unique physical, chemical, and electronic properties of GNPs, in combination with high electrical conductivity and biocompatibility make them a more effective interface for electrical stimulation [31]. As an electrically conductive matrix, GNP coated surface allowed enhanced neurite outgrowth of PC12 cells, when stimulated with alternating current [21]. Furthermore, cellular uptake of these electroactive GNPs can act as a useful tool for the control and manipulation of MSC differentiation pathways under electrical stimulation [32,33].

In the above perspective, we present an inventive strategy to trigger the selective differentiation of hMSCs using electrically driven GNPs. Specifically, an uniform layer of GNPs electrostatically embedded onto thin polyaniline (PANI) films was employed as an extracellular conduit to deliver electric stimuli to adhered hMSCs. Concurrently, hMSCs were also internalized with monodispersed GNPs so as to enable EF-induced physical stresses intracellularly. In particular, we investigated whether it is possible to differentiate GNP internalized hMSCs to neural or cardiomyogenic-like cells, respectively by employing two different EF stimulation modalities comparable with those detected *in vivo* in nerve and heart tissues. The ability of such novel nano-biomaterial based cell modification approach to elicit differentiation of hMSCs to electrically excitable cells in response to exogenous EFs has been previously unexplored. The present work therefore demonstrates the first results of such novel biophysical stimulation approach for hMSC differentiation.

2. Materials and methods

2.1. Synthesis and physicochemical characterization of GNPs

Approximately 20 nm sized gold nanoparticles were synthesized by the reduction of tetrachloroauric (III) acid (Alpha aeser) using trisodium citrate, as described in the literature [30,34]. The size and Zeta potential of citrate stabilized GNPs was determined using ZetaPALS Zeta potential & particle size analyzer (Brookhaven Instrument Corp., NY). For the transmission electron microscopic (TEM, JEOL JEM-2100F) analysis of GNP size and shape, the diluted gold colloid was drop casted on Fomvar coated copper grids. The size distribution of GNPs was determined by measuring at least 400 particles from different regions of the grid using ImageJ and Origin software. The UV–visible absorption spectra were collected using Eppendorf BioSpectrometer® in the range of 400–800 nm.

As-prepared gold colloids were surface modified with 2-Mercaptosuccinic Acid (MSA) for the layer by layer assembly of films. The modification was performed under stirring at 50 °C for 8–12 h by mixing a certain volume of as-prepared gold colloids with an aqueous solution containing a large excess of MSA, as reported elsewhere [35]. The thiol was introduced as its sodium salt by stoichiometrical neutralization with sodium hydroxide. Both citrate stabilized as well as MSA capped GNPs were centrifuged for 30 min at 15000 rpm to remove the excess reagents. The citrate stabilized GNPs were then resuspended in complete αMEM medium and used for cell culture experiments. Likewise, the MSA-capped GNPs were utilized for the layer by layer assembly of nanocomposite films.

2.2. Fabrication of multilayered films of PANI-GNP

Five precursor bilayers of PANI/GNP were deposited by sequential dipping of glass substrates in polyelectrolyte solutions [44]. Prior to coating, glass substrates were hydroxylated in piranha solution for 1 h (7:3 v/v concentrated sulfuric acid/hydrogen peroxide solution) and in 1:1:5 ammonium hydroxide/hydrogen peroxide/water for another hour. Further treatment was performed to make the glass substrate positively charged by immersing it in the solution of toluene with 5% of (*N*-2-aminoethyl-3-aminopropyl) trimethoxysilane for 24 h. It was rinsed sequentially with toluene, methanol/toluene, and then toluene before being washed several times in deionized water.

A thin layer of polystyrene sulphonate (PSS) was deposited initially by dipping the substrates in 2 mg/mL PSS in water with a pH adjusted to 2.8 [32,36]. An alternate layer of PANI was then coated by immersing the substrates in PANI solution for 15 min. PANI was prepared by the oxidative polymerization of aqueous solutions of aniline by ammonium peroxydisulfate (APS) at −30 °C [33,37]. The purified PANI was then dissolved in dimethyl acetamide (DMAc) at a concentration of 20 mg/mL. After filtration and stirring for 24 h, it was diluted 10 times with water and the pH was slowly lowered to 2.8. This diluted PANI solution was used further for the coating experiments. After coating PANI layer, the substrate was washed in MilliQ water with pH 2.5, dried and subsequently immersed in 1 mg/mL GNP-MSA aqueous solution. This process was repeated until the required number of layers was obtained.