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Effects of two-dimensional materials on human mesenchymal stem cell behaviors

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ABSTRACT

Graphene, a typical two-dimensional (2D) material, is known to affect a variety of stem cell behaviors including adhesion, spreading, growth, and differentiation. Here, we report for the first time the effects of four different emerging 2D materials on human adipose-derived mesenchymal stem cells (hADMSCs). Graphene oxide (GO), molybdenum sulfide (MoS_2), tungsten sulfide (WS_2), and boron nitride (BN) were selected as model two-dimensional materials and were coated on cell-culture substrates by a drop-casting method. Acute toxicity was not observed with any of the four different 2D materials at a low concentration range (<5 μg/ml). Interestingly, the 2D material-modified substrates exhibited a higher cell adhesion, spreading, and proliferation when compared with a non-treated (NT) substrate. Remarkably, in the case of differentiation, the MoS_2-, WS_2-, and BN-modified substrates exhibited a better performance in terms of guiding the adipogenesis of hADMSCs when compared with both NT and GO-modified substrates, based on the mRNA expression level (qPCR) and amount of lipid droplets (ORO staining). In contrast, the osteogenesis was found to be most efficiently induced by the GO-coated substrate (50 μg/mL) among all 2D-material coated substrates. In summary, 2D materials could act as favorable sources for controlling the stem cell growth and differentiation, which might be highly advantageous in both biomedical research and therapy.

1. Introduction

Stem cells have emerged in the field of regenerative medicine mostly owing to their unique ability to generate every type of cell composing the human body [1–4]. Conventionally, the differentiation of stem cells is known to be controlled by the composition of the conditioned medium that contains several differentiation factors including biomolecules (e.g., ascorbic acid, dexamethasone, and β-glycerophosphate) and proteins (e.g., CHIR, insulin, and bone morphogenetic protein) [5–9]. However, besides these soluble factors, recent findings suggest that biophysical cues, which encompass the physical and mechanical properties of the underlying substrates wherein the cells are attached and grow, could also play a key role in guiding the differentiation of stem cells into specific lineages. A biophysical stimulus is generally governed by the cell-substrate interactions, which ultimately result in the alteration of both cytoskeletal dynamics and gene/protein expression of the cells. Nanostructures, micropatterns, and nanomaterials have recently been reported to be successful for steering stem cell differentiation, in the presence/absence of soluble differentiation factors [10–14]. Among such materials, graphene, a typical two-dimensional (2D) material that consists of carbon atoms arranged in a perfect honeycomb structure, has recently gained remarkable attention as an effective biophysical stimulator for cells, especially for stem cell differentiation [4,15]. Several differentiations such as adipogenesis, osteogenesis, chondrogenesis, and neurogenesis of mesenchymal stem cells (MSCs) or neural stem cells (NSCs) were studied in the presence of graphene derivatives [16–18]. Among these, adipogenesis and osteogenesis of MSCs were found to be effectively controlled by graphene and graphene oxide (GO), respectively, due to their influence on both the absorption of

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differentiation factors (e.g., dexamethasone, β-glycerophosphate, and ascorbic acid) and cell adhesion/spreading [19–22].

Besides graphene derivatives, other types of two-dimensional materials with a thickness of a few nanometers such as transition-metal dichalcogenides (TMDs) have attracted considerable attention owing to their distinct characteristics and applications. Molybdenum sulfide (MoS_2), tungsten sulfide (WS_2), and boron nitride (BN) have been mostly studied due to their mechanical and electronic properties [23]. MoS_2 is classified as belonging to the TMDs family, whose major characteristics are a high conductivity owing to its anisotropic layered structure, direct bandgap due to its mono- and few-layers, and ability to form 2D layers by strong covalent bonding among the composing atoms [24–27]. Bulk MoS_2 consists of monolayers, which are held together through Van der Waals interactions, and has an indirect bandgap [27–30]. Bulk WS_2 forms hexagonal crystals with a layered structure that relates to that of MoS_2. Its monolayer structure has recently been under particular investigation for many applications, e.g., lithium ion battery production, key role of hydrogen evolution catalysis, etc. [31,32]. On the other hand, BN is a preeminent graphene-like refractory compound owing to its thermal and chemical stability [33]. Unlike graphene-based materials, the applications of other types of 2D materials are mostly limited to the electronic and optoelectronic areas (e.g., electronic circuits, photovoltaics, displays, and energy storage devices). However, considering the discovery of graphene and its application trends, ranging from electronic and optical to various biomedical applications (e.g., biosensors, bioelectronics, cancer therapy, and stem cell research), the potential of two-dimensional materials in the biomedical field should also be properly investigated, since it might open new applicative venues for such materials.

In this study, we examined the effects of 2D materials on human mesenchymal stem cells (hADMSCs). In particular, GO, MoS_2, WS_2, and BN were chosen as model 2D materials and further coated on the cover glass. After the successful deposition of these 2D materials on the substrate achieved by varying their concentrations via Raman spectroscopy, the biocompatibility of the four different 2D material-modified substrates was first investigated to find a suitable surface coverage (non-toxic conditions). Subsequently, hADMSCs were seeded on each substrate and their behaviors, such as cell-substrate interactions, and cell spreading and proliferation were characterized by SEM imaging and actin/DAPI staining. Thereafter, the effects of the 2D materials on the differentiation of hADMSCs were intensively analyzed to determine the potential of these four selected 2D materials for stem cell applications. The adipogenesis of hADMSCs on each substrate was characterized by checking the mRNA expression level of lipoprotein lipase (LPL) as well as the lipid droplet formation. In the case of the osteogenesis, the expression levels of two major bone matrix genes, the Runt-related transcription factor 2 (RUNX2) and alkaline phosphatase (ALP), and the osteoblast mineralization level were analyzed.

2. Materials and methods

2.1. Fabrication and Raman characterization of 2D material-modified substrates

The glass substrates were washed with a 1% Triton X-100 solution, deionized (DI) water, and 70% ethanol in an ultrasonic bath. After washing and drying, the substrates were immersed in a 5 mM APTES solution for 30 min at room temperature, and then washed with DI water. The APTES-treated glass substrates were dried, and then subjected to the drop casting method to produce 2D material films. The 2D material solutions used were: GO solution (500 mg/L), BN solution (5.4 mg/L), MoS_2 solution (18 mg/L), and WS_2 solution (26 mg/L). Next, 200 μl of solution was dropped on the treated-glass substrates, followed by evaporation at 70 °C. The Raman spectra of the 2D materials were obtained using XperRam200 (Nanobase Co., Ltd, South-Korea). A DPSS laser (532 nm) was used as light source, while the excitation light was directed on the sample using a 40× microscope objective with an exposure time of 1000 ms and laser power of 1 mV.

2.2. Determination of the cytotoxic effect of 2D material-modified substrates

Substrate modifications were obtained using various concentrations of 2D material solutions as follows: GO at a concentration of 100, 200, 300, 400, and 500 μg/ml; MoS_2 at a concentration of 1, 4, 8, 12, and 18 μg/ml; WS_2 at a concentration of 1, 5, 10, 20, and 25 μg/ml; BN at a concentration of 1, 2, 3, 4, and 5 μg/ml. The P5 passages of hADMSCs were seeded on the 2D material-modified substrates at a density of 5×10^3 cells. The cell viability was assessed after 2 and 7 days of treatment by using a CCK-8 cell viability assay (Dojindo Laboratories, Japan). The survival rate could be calculated by comparing the amount of cells cultured on the modified substrates with cells cultured on an unmodified substrate.

2.3. Cell proliferation and morphological analysis

The hADMSCs grown on the 2D material-modified substrates were washed with phosphate buffered saline (PBS) and subsequently fixed with a 4% formaldehyde solution in PBS for 10 min at room temperature. After fixation, actin staining was performed using Alexa Fluor 568 phalloidin (Invitrogen) to visualize the cell morphology and spreading; then, the cells were counterstained with Hoechst 33342 (Thermo Scientific) diluted in PBS to label the DNA according to the manufacturer's protocol. SEM images were obtained using a ZEISS SIGMA field emission scanning electron microscope (FE-SEM, Carl Zeiss Microscopy Co.) in order to investigate the cell morphology and cell-2D material interactions.

2.4. hADMSCs differentiation and staining assay

The hADMSCs were grown for 2 days on each 2D material-modified substrate, followed by differentiation into osteogenic and adipogenic lineages. For adipogenesis, the cells were induced using the StemPro Adipogenesis differentiation kit media (Gibco), while the osteogenesis was induced by using hADMSC growth media supplemented with 50 μM ascorbic acid, 10 mM β-glycerophosphate, and 100 nM dexamethasone. The differentiation medium was subsequently changed every 3 days. After 21 days, ORO staining was carried out to assess the lipid/fatty acid droplets in the adipocytes, while ARS staining was performed to evaluate the calcium phosphate formation.

2.5. Quantitative real-time RT-PCR analysis

RNA was extracted using the RNeasy Mini Kit (Qiagen) and reverse transcribed to complementary DNA (cDNA) using Superscript II Reverse Transcriptase (Invitrogen). Real-time PCR was performed using SYBR *Premix Ex Taq* (Tli RNaseH Plus, TaKaRa) to detect gene expression indicative of osteogenesis and adipogenesis. The thermocycling conditions used were as follows: 95 °C for 2 min, 95 °C for 3 s, and 60 °C for 30 s for a total of 40 cycles. The relative mRNA expression of each gene compared with the GAPDH gene was calculated and normalized relative to the value of cells cultured on the unmodified glass substrate. The primers that were used can be seen on supplementary information.

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