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Graphene enhances the cardiomyogenic differentiation of human embryonic stem cells

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ABSTRACT

Graphene has drawn attention as a substrate for stem cell culture and has been reported to stimulate the differentiation of multipotent adult stem cells. Here, we report that graphene enhances the cardiomyogenic differentiation of human embryonic stem cells (hESCs) at least in part, due to nanoroughness of graphene. Large-area graphene on glass coverslips was prepared via the chemical vapor deposition method. The coating of the graphene with vitronectin (VN) was required to ensure high viability of the hESCs cultured on the graphene. hESCs were cultured on either VN-coated glass (glass group) or VN-coated graphene (graphene group) for 21 days. The cells were also cultured on glass coated with Matrigel (Matrigel group), which is a substrate used in conventional, directed cardiomyogenic differentiation systems. The culture of hESCs on graphene promoted the expression of genes involved in the stepwise differentiation into mesodermal and endodermal lineage cells and subsequently cardiomyogenic differentiation compared with the culture on glass or Matrigel. In addition, the culture on graphene enhanced the gene expression of cardiac-specific extracellular matrices. Culture on graphene may provide a new platform for the development of stem cell therapies for ischemic heart diseases by enhancing the cardiomyogenic differentiation of hESCs.

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1. Introduction

Due to the unique physical and chemical properties of graphene, this material has been reported to act as a culture substrate that promotes the lineage specification of adult mesenchymal stem cells (MSCs) and neural stem cells, both of which are multipotent stem cells. The culture of human neural stem cells (hNSCs) on graphene promotes their differentiation toward neurons through electrical stimulation facilitated by graphene [1,2]. Graphene can also promote the adhesion of MSCs [3], and the culture of MSCs on graphene enhances their osteogenic differentiation through strong cell adhesion to graphene [4]. Graphene has also been used for the culture of pluripotent stem cells. Graphene maintains the pluripotency of mouse induced pluripotent stem cells [5]. Human embryonic stem cells (hESCs) adhered to Geltrex®-coated graphene remained viable and pluripotent and proliferated [6]. However, the effect of graphene on the differentiation of pluripotent ESCs has not yet been reported.

Cardiomyocytes generated from stem cells would be a useful cell source for cell-based therapies for ischemic heart diseases. A variety of adult stem cells, such as bone marrow-derived stem cells [7], adipose-derived stem cells [8], resident cardiac stem cells [9], and umbilical cord blood stem cells [10], have been used to treat ischemic heart diseases. However, these adult stem cells are known to have limited ability to differentiate into cardiomyocytes [11]. In contrast, ESCs are known to differentiate spontaneously into cardiomyocytes [12]. When these cells are transplanted into a damaged heart, ESCs integrate into the recipient heart and improve the heart functions [13]. Therefore, hESCs would be a useful cell-source for stem cell-based therapy for ischemic heart diseases.

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Although graphene exerts stimulatory effects on the lineage specification of multipotent adult stem cells, its effects on the differentiation of pluripotent ESCs have not yet been reported. In the present study, we investigated whether culture on graphene enhances the cardiomyogenic differentiation of hESCs. Large-area graphene on glass coverslips was prepared through the chemical vapor deposition method. The spontaneous cardiomyogenic differentiation of hESCs was investigated by culturing hESCs on either glass (control) or graphene without the addition of exogenous chemicals for differentiation induction and by evaluating the expression of cardiac-specific genes. The differentiation of these cells was also compared with that of cells cultured on Matrigel-coated glass, which is a substrate used in the conventional, directed two-dimensional culture differentiation systems for the cardiomyogenic differentiation of hESCs without forming embryoid bodies [14]. This culture system showed an efficient differentiation into cardiomyocyte phenotype [14]. The mesodermal and endodermal differentiations were compared among the experimental groups because these differentiations are known to be intermediate stages of the cardiomyogenic differentiation of hESCs [14]. The mechanisms underlying the enhanced cardiomyogenic differentiation were also investigated.

2. Materials and methods

2.1. Graphene preparation and characterization

Monolayer graphene was synthesized through the chemical vapor deposition (CVD) process and transferred to glass coverslips as previously described [2]. The graphene was sterilized by UV treatment for 30 min prior to cell culture. The structural properties of the graphene on the glass coverslip were further investigated through Raman spectroscopy (RM 1000-Invia, Renishaw, UK). The optical transmittance of the graphene on the coverslip was measured using an ultraviolet–visible spectrometer (UV-3600, Shimadzu, Japan). TEM and selected area electron diffraction analyses were conducted on a TEM (JEOL 2100, JEOL, Japan) operated at 200 kV. The rotation between the TEM images and the corresponding SAED patterns was calibrated using molybdenum trioxide crystals. The sheet resistances of the graphene on the coverslip were measured through the van der Pauw four-probe method using a Hall measurement system. The surface morphology of graphene and glass was examined with non-contact mode AFM (XE-100 system, Park Systems, Suwon, Korea).

2.2. hESC culture

SNUhES31 (Institute of Reproductive Medicine and Population, Medical Research Center, Seoul National University, Seoul, Korea), which is a hESC line, was maintained in their undifferentiated state by feeder-free culturing on human recombinant vitronectin (VN, Life Technologies, Carlsbad, CA, USA)-coated (0.5 $\mu g/cm^2$) culture dishes with Essential 8™ medium (Life Technologies) as previously described [15]. The culture medium was changed daily, and the hESCs were passaged every week.

2.3. Differentiation of hESCs into cardiomyocytes using a two-dimensional system

For the cardiomyogenic differentiation of hESCs using a two-dimensional system, cultured hESC colonies were fragmented into small clumps using the STEMPRO® EZPassage tool (Life Technologies). Prior to cell plating, glass coverslips were coated with Matrigel (BD Bioscience, San Jose, CA, USA) [16] or VN [17]. Graphene was also coated with VN. The Matrigel- or VN-coated glass and VN-coated graphene were placed onto six-well plates. Small clumps of hESCs were plated on each of the Matrigel- or VN-coated glass and VN-coated graphene (150 clumps per well). The in vitro cardiomyogenic differentiation was performed as previously described [14]. Briefly, the attached hESCs were expanded in hESC media composed of Dulbecco's Modified Eagle's Medium (DMEM)/F12 (Gibco BRL, Gaithersburg, MD, USA) supplemented with 20% (v/v) knockout serum replacement (Life Technologies), 4 ng/ml FGF2 (R&D Systems, Minneapolis, MN, USA), 1% nonessential amino acid (Life Technologies), 0.1 mM β-mercaptoethanol (Sigma), and 0.2% primocin (InvivoGen, San Diego, CA, USA) for 4 days and then cultured in hESC media without FGF2 for 3 days. The cells were cultured in DMEM (Gibco BRL) containing 10% (v/v) fetal bovine serum (FBS, Gibco BRL) for 7 days. Finally, the cells were cultured in DMEM containing 20% (v/v) FBS for 7 days. The culture media were changed every 24 h.

2.4. Viability of hESCs cultured on graphene

The live and dead cells on non-coated graphene or VN-coated graphene were detected with fluorescein diacetate (FDA,

Table 1
Human-specific primers for each gene.

Gene	Primer
GAPDH	Sense 5′-GTC GGA GTC AAC GGA TTT GG-3′ Antisense 5′-GGG TGG AAT CAA TTG GAA CAT-3′
BRACHYURY (T)	Sense 5′-CAG TGA CTT TTT GTC GTG GCA-3′ Antisense 5′-CCA ACT GCA TCA TCT CCA CA-3′
MESP1	Sense 5′-TGT GAG CAC CGA GGC TTT TT-3′ Antisense 5′-TCC TGC TTG CCT CAA AGT GT-3′
SOX17	Sense : 5′-AAC TGG TTG GCT TGT CAT GAG-3′ Antisense 5′-TAC TTC CAA GGA ACT GCA TGG-3′
FOXA2	Sense 5′-CCA TTG CTG TTG TTG CAG GGA AGT-3′ Antisense 5′-CAC CGT GTC AAG ATT GGG AAT GCT-3′
TUBB3	Sense 5′-TTC CTG CAC TGG TAC ACG G-3′ Antisense 5′-TGC GAG CAG CTT CAC TTG-3′
NKX2-5	Sense 5′-GCA GAG ACC TCC CGT TTT GTT-3′ Antisense 5′-GCC ACC GAC ACG TCT CAC T-3′
MEF2C	Sense 5′-CCT GCA AAT ATG GCC CTA GAA-3′ Antisense 5′-CGG GAT TGT TCA ACA GTC CTA-3′
α-MHC	Sense 5′-GCC CCG CCC CAC AT-3′ Antisense 5′-CCG GAT TCT CCC GTG ATG-3′
β-MHC	Sense 5′-CCA CCC AAG TTC GAC AAA ATC-3′ Antisense 5′-CGT AGC GAT CCT TGA GGT TGT A-3′
MLC2a	Sense 5′-CCC CAG CGG CAA AGG-3′ Antisense 5′-CCA CCT CAG CTG GAG AGA ACT T-3′
cTnT	Sense 5′-CAG GAT CAA CGA TAA CCA GAA AGT C-3′ Antisense 5′-GTG AAG GAG GCC AGG CTC TA-3′
CONNEXIN43	Sense 5′-ACT GGC GAC AGA AAC AAT TCT TC-3′ Antisense 5′-TTC TGC ACT GTA ATT AGC CCA GTT-3′
COLLAGEN TYPE I	Sense 5′-CAG CCG CTT CAC CTA CAG C-3′ Antisense 5′-TTT TGT ATT CAA TCA CTG TCT T-3′
COLLAGEN TYPE III	Sense 5′-GGG AAT GGA GCA AAA CAG TCT T-3′ Antisense 5′-CCA ACG TCC ACA CCA AAT TCT-3′
COLLAGEN TYPE IV	Sense 5′-TGT CCA ATA TGA AAA CCG TAA AGT G-3′ Antisense 5′-CAC TAT TGA AAG CTT ATC GCT GTC TT-3′
FIBRONECTIN	Sense 5′-TCC ACG GGA GCC TCG AA-3′ Antisense 5′-ACA ACC GGG CTT GCT TTG-3′
LAMININ	Sense 5′-CAC AAC AAC ATT GAC ACG ACA GA-3′ Antisense 5′-GCT GGA GGG CAT CAC CAT AGT-3′

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