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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.

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 μg/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′

Sigma–Aldrich, St. Louis, MO, USA) and ethidium bromide (EB, Sigma) at day 2. The dead cells were stained orange due to the nuclear permeability of EB. The viable cells, which are capable of converting the non-fluorescent FDA into fluorescein, were stained green.

2.5. Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

The mRNA expression levels of differentiated hESCs were analyzed through qRT-PCR. The total RNA was extracted from the differentiated hESCs on days 4, 7, 14, and 21 and reverse-transcribed into cDNA ($n = 4$ for each group per time point). In the glass and graphene groups, coverslips were detached from the well and then total RNA was extracted. The qRT-PCR was performed using the StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA) with a FAST SYBR Green PCR master mix (Applied Biosystems). Each of the 45 cycles performed consisted of the following temperature program: 94 °C for 3 s and 60 °C for 30 s. The primer sequences are shown in Table 1.

2.6. Western blot assay

hESCs cultured for 21 days on Matrigel- or VN-coated glass and VN-coated graphene were lysed with sodium dodecyl-sulfate (SDS) sample buffer [62.5 mM Tris–HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 50 mM dithiothreitol, and 0.1% (w/v) bromophenol blue, $n = 3$ for each group]. Western blot analysis was performed through 10% (w/v) SDS–polyacrylamide gel electrophoresis. The proteins were probed with antibodies against human vinculin (Abcam, Cambridge, UK), paxillin (Abcam), FAK (Abcam), pFAK (Abcam), ERK 1/2 (Abcam), and pERK 1/2 (Abcam). The blot data were quantified through densitometric scanning (Image-Pro Plus software, Media Cybernetics, Marlow, UK).

2.7. Statistical analysis

The quantitative data are expressed as the means ± standard deviation. The statistical analysis was performed by analysis of variance (ANOVA) using a Bonferroni test. A p value <0.05 was considered statistically significant.

3. Results and discussion

The large-area, monolayer graphene used in this study was synthesized on copper foils through the CVD method. To confirm the presence of graphene, the graphene was transferred onto a SiO_2/Si substrate according to a previously reported method [18]. The uniform color contrast of the optical micrograph indicates that the graphene film has excellent thickness uniformity (Fig. 1A). Optical images of the glass coverslip and the graphene-coated glass coverslip showed that both were transparent (Fig. 1B). The contact angle measurements showed that the graphene-coated glass coverslip was slightly hydrophobic (74.4 ± 3.5°), whereas the contact angle of the coverslip was 64.2 ± 3.7° (Fig. 1C). The Raman spectrum of graphene showed three main characteristic peaks; D, G, and 2D peaks. The G peak, D band, and 2D peak showed at near 1580 cm^{-1}, 1350 cm^{-1}, and near 2700 cm^{-1}, respectively. The intensity of the I_{2D}/I_G ratio is more than threefold higher than that G peak, and there is no measurable peak observed at ~1350 cm^{-1} (the D band) (Fig. 1D), which indicates the synthesis of high-quality monolayer graphene [2]. In addition, the graphene-coated glass coverslip exhibited high transparency with a transmittance that exceeded 97.4% at a wavelength of 550 nm (Fig. 1E), which confirms the presence of uniform and monolayer graphene [2]. Fig. 1G shows a representative low-magnification TEM image of graphene sheet on a TEM grid. The typical SAED pattern for well-synthesized graphene was observed for the graphene used in this study (Fig. 1F and G). The transferred graphene on the coverslip

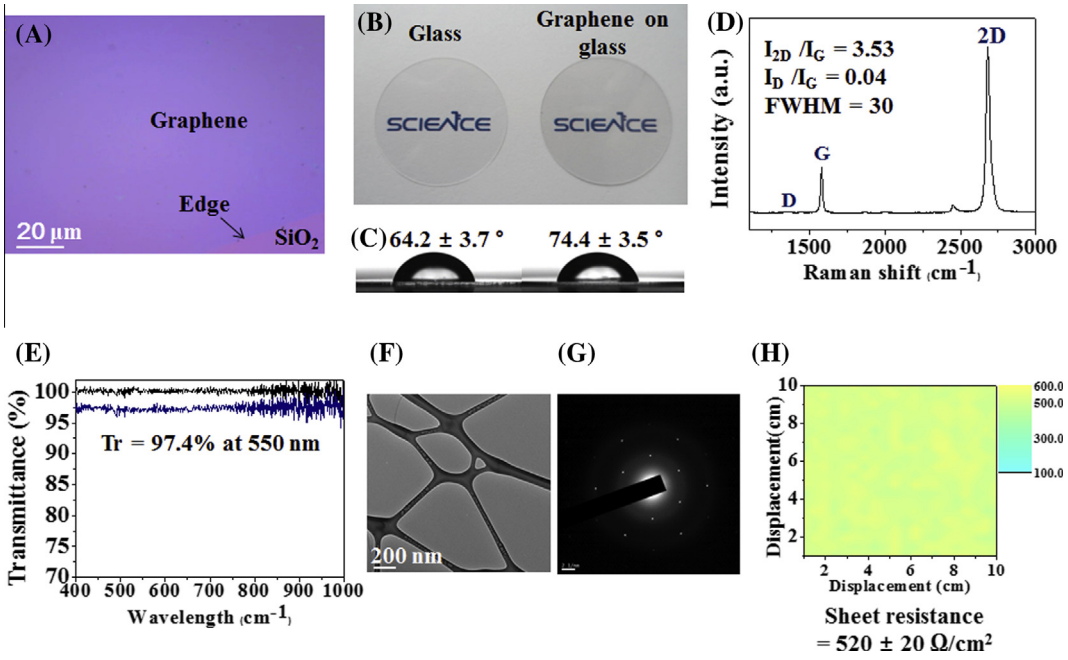

Fig. 1. Preparation and characterization of large-area graphene substrates. (A) A typical optical microscopic image of graphene transferred onto 300-nm-thick SiO_2/Si substrate. (B) Photograph and (C) contact angle of a glass coverslip and graphene on a glass coverslip. (D) Raman spectroscopy of the transferred graphene. (E) UV–visible spectra of transferred graphene on a glass coverslip and a glass coverslip substratum, showing the transparency of the graphene on the coverslip. Black and blue colors indicate glass coverslip color and graphene on a glass coverslip, respectively. (F) TEM image of graphene on a TEM grid. (G) SAED pattern. (H) Sheet resistance of monolayer graphene on a glass coverslip.

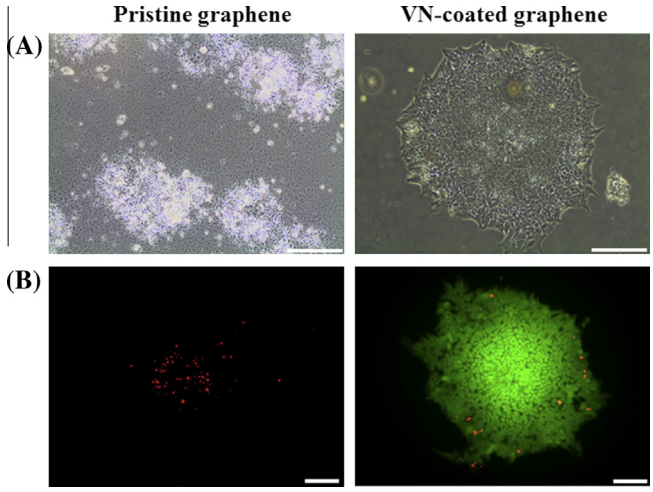

Fig. 2. The coating of graphene with VN is required to achieve a high viability of hESCs cultured on the graphene. hESCs were cultured on either pristine graphene or VN-coated graphene for 2 days. (A) Light microscopic images of the hESCs. (B) Fluorescence microscopic images of the hESCs stained with FDA and EB. The green and orange-red colors indicate viable and dead cells, respectively. The scale bars indicate (A) 200 μm and (B) 100 μm. The images A and B were taken from different areas of the culture dishes.

showed a sheet resistance of $520 \pm 20\,\Omega/cm^2$ with a high uniformity (Fig. 1H) [2].

Prior to culture for cardiomyogenic differentiation, we tested whether hESCs could adhere to graphene and survive. hESC clumps were plated on pristine graphene and VN-coated graphene. Two days later, the hESCs on VN-coated graphene showed good cell-attachment and colony formation (Fig. 2A). In contrast, the hESCs did not adhere to pristine graphene. The viability of hESCs cultured on pristine graphene and VN-coated graphene was evaluated by live and dead assays with FDA/EB staining (Fig. 2B). The hESCs on pristine graphene were dead (orange-red color), whereas most of the hESCs cultured on VN-coated graphene were viable (green color, Fig. 2B). Thus, VN-coated graphene was used for the hESC culture in the following experiments.hESCs first differentiate into mesodermal cells and endodermal cells, and the mesodermal cells further differentiate into cardiogenic lineages in two-dimensional, directed differentiation systems [14]. hESCs were cultured on Matrigel-coated glass (Matrigel group), VN-coated glass (glass group), or VN-coated graphene (graphene group) for 21 days, and the mesodermal, cardiac mesodermal, endodermal, and ectodermal gene expression profiles were determined by qRT-PCR assay. On day 14, the graphene group showed a significantly enhanced expression of mesodermal genes (T and M-CAD) and a cardiac mesodermal gene (MESP1) compared with both the Matrigel and the glass groups (Fig. 3A and B). T expression is known to induce the expression of MESP1 [19]. MESP1 is a master regulator of cardiac progenitor specification and is needed to enter the cardiac mesodermal stage [20–22]. The graphene group showed higher expression of FOXA2 on day 14 and SOX17 on day 14 and 21, both of which are endodermal genes, compared with the Matrigel and the glass groups (Fig. 3C). In contrast, the ectodermal gene (TUBB3) expression was not increased in the graphene group compared with the Matrigel and the glass group throughout the culture period (Fig. 3D). These results suggest that graphene promotes mesodermal lineage differentiation at the middle-stage time point and differentiation into the cardiac mesodermal lineage, which is a more cardiogenic lineage than the mesodermal lineage, at the end-stage time point.

The endodermal lineage induction by graphene may promote the cardiomyogenic differentiation of hESCs. Signals from the endoderm, such as bone morphogenetic protein (BMP), and transforming growth factor-β and fibroblast growth factor (FGF), are important for cardiac development to induce the cardiac differentiation of murine ESCs and heart development [23–25]. It has also been reported that co-culture with an endodermal cell line induces the cardiac differentiation of hESCs [26]. In two-dimensional culture systems, the presence of endodermal cells around the mesodermal cells appears to induce the cardiomyogenic differentiation of hESCs [14]. Therefore, the enhanced endodermal lineage differentiation by graphene (Fig. 3C) may induce an enhancement in the cardiomyogenic differentiation of hESCs.

We investigated whether the culture of hESCs on graphene promotes their cardiomyogenic differentiation by evaluating the cardiomyogenic gene expression through a qRT-PCR assay. Strikingly, the expression of many cardiomyogenic markers was enhanced in the graphene group compared with both the Matrigel and the glass groups, even in the absence of cardiomyogenic inducers in the culture medium (Fig. 3E). The gene expression of cardiomyogenic transcriptional factors (NKX2-5, GATA4, and MEF2C), cardiomyogenic contractile proteins (α-MHC, β-MHC, MLC2a, and cTnT), and gap junction proteins (CONNEXIN43) was significantly higher in the graphene group compared with both the Matrigel and the glass groups on days 14 and/or 21. Early cardiomyocyte marker (α-MHC and β-MHC) expression was maximal on day 14 and decreased on day 21 in the graphene group, whereas late cardiomyocyte marker (MLC2a, and cTnT) expression increased with the culture time from day 14 to day 21. To further investigate the effects of graphene on the cardiomyogenic differentiation of hESCs, the gene expression of cardiac-specific ECMs was evaluated through a qRT-PCR assay. Cardiomyocytes derived from hESCs were reported to be encased in a network of fibronectin, laminin, and collagen, which are the major components of the cardiac ECMs [27,28]. The gene expression of cardiac-specific ECMs, such as COLLAGEN TYPE I, COLLAGEN TYPE III, COLLAGEN TYPE IV, FIBRONECTIN, and LAMININ, was enhanced on day 21 in the graphene group compared with both the Matrigel and the glass groups (Fig. 3F).

Also, the nanoroughness of graphene may contribute to the enhanced cardiomyogenic differentiation by enhancing hESC adhesion and the upregulation of extracellular signal-regulated kinase (ERK) signaling. Graphene showed larger nanoroughness than glass (Fig. 4A). It was reported that nanoroughness of graphene was produced by wrinkles that were formed in the process of graphene coating on support surfaces such as copper and glass [29]. Graphene enhanced hESC adhesion, as the expression of phosphorylated focal adhesion kinase (pFAK), vinculin, and paxillin, which are related to the focal adhesion of cells [30,31], was enhanced in the graphene group compared with the other groups (Fig. 4B). The enhanced hESC adhesion on graphene with nanoroughness features is consistent with the results of previous studies in which cell adhesion was enhanced on substrates with nanoscale protrusions [32,33]. Graphene also enhanced phosphorylation of ERK compared with both the Matrigel and the glass groups (Fig. 4B). Strong hESC adhesion to graphene may be responsible for the enhanced cardiomyogenic differentiation through FAK and ERK signaling. It has been shown that stem cell differentiation is enhanced on nanoroughness substrates through FAK [34]. FAK gene-inactivated mice showed major defects in the axial mesodermal tissues and cardiovascular system [35,36]. Activation of ERK enhances the cardiomyogenic differentiation of hESCs [37]. In addition, nano-structured surfaces enhanced adhesion, proliferation, migration and cardiomyogenic differentiation of adult cardiac progenitor cells [38]. Therefore, nanoroughness of graphene would be one of possible factors that enhance cardiomyogenic differentiation of hESCs.

In study, we used VN coating on graphene for hESC adhesion because it was previously reported that VN enhanced endoderm

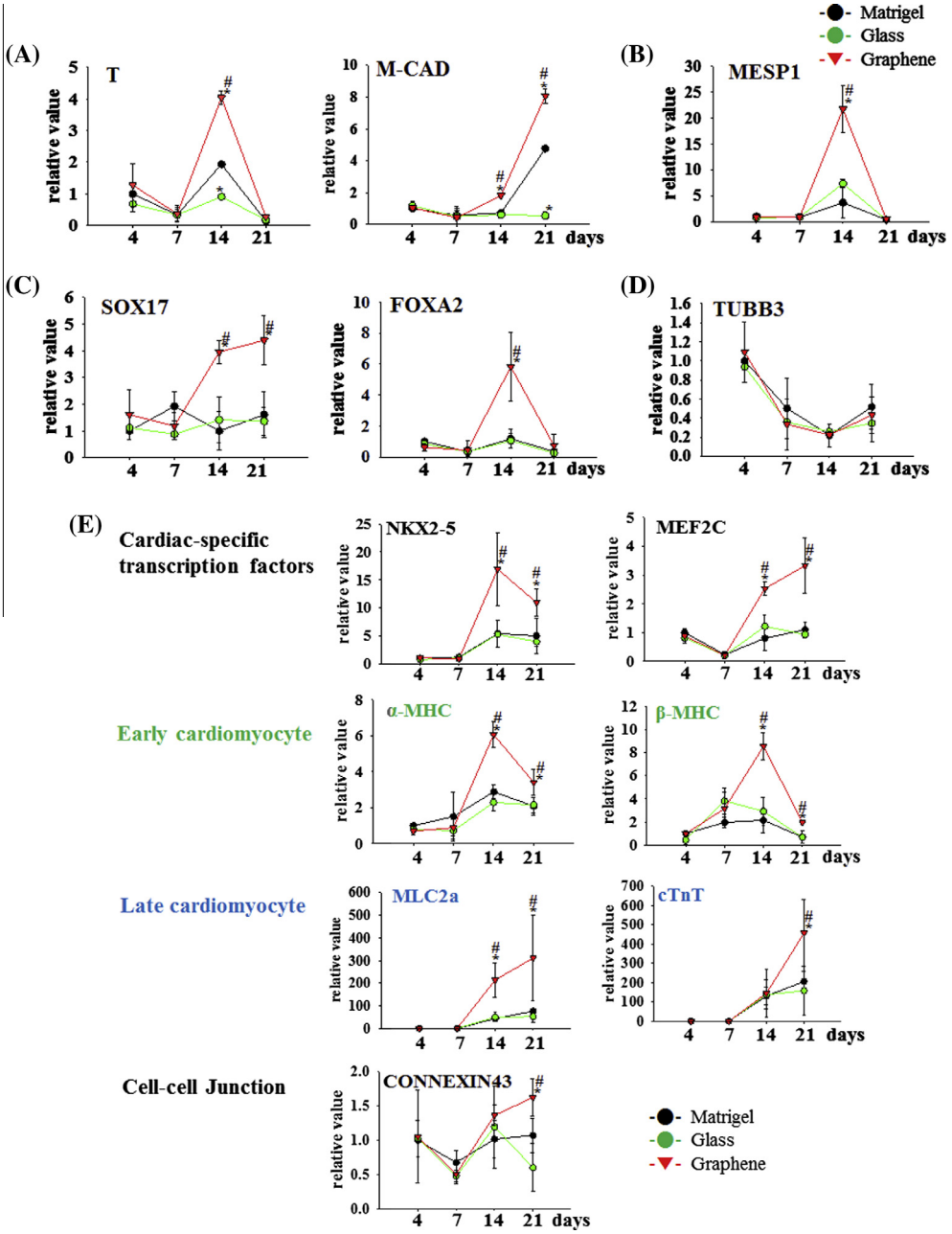

Fig. 3. Culture on graphene enhances expression of the mesodermal, endodermal lineage, cardiomyogenic, and cardiac-specific ECM genes in hESCs. (A) The mesodermal, (B) cardiac mesodermal, (C) endodermal, (D) ectodermal, (E) cardiomyogenic, and (F) ECM gene expression profiles of hESCs cultured for 21 days on Matrigel-coated glass (Matrigel), VN-coated glass (glass), or VN-coated graphene (graphene), as determined by qRT-PCR assay. The values were normalized to the levels obtained for the Matrigel group ($n = 4$ per group, $^{*}p < 0.05$ versus Matrigel, $^{\#}p < 0.05$ versus glass).

differentiation of hESCs via VN-binding integrin αV [39], and that culture of murine ES cells on VN-coated scaffolds increased the differentiation into cardiac progenitor cells [40]. However, there was no difference in expressions of endodermal and cardiomyogenic genes between the (VN-coated) glass group and (VN-coated) graphene group in the present study. Therefore, the enhanced endodermal and cardiomyogenic gene expression in the graphene group may result from the unique properties of graphene.

In summary, the culture of hESCs on graphene promotes the stepwise differentiation of these cells into mesodermal cells and endodermal cells and their subsequent cardiomyogenic differentiation compared with their culture on glass. Moreover, the analysis of the cardiomyogenic differentiation of hESCs cultured on graphene or Matrigel revealed that graphene was superior to Matrigel, which is a substrate used in conventional cardiomyogenic differentiation systems. The graphene-enhanced cardiomyogenic differentiation may be, at least in part, due to nanoroughness of graphene, which enhanced hESC adhesion and the upregulation of ERK signaling. However, we did not observe beating cells following induction of cardiomyogenic differentiation with graphene. This indicated that our method does not induce full differentiation into contractile cardiomyocytes. The data suggest that graphene likely promotes the adsorption and correct presentation of vitronectin, which subsequently supports expression of cardiomyogenic genes.

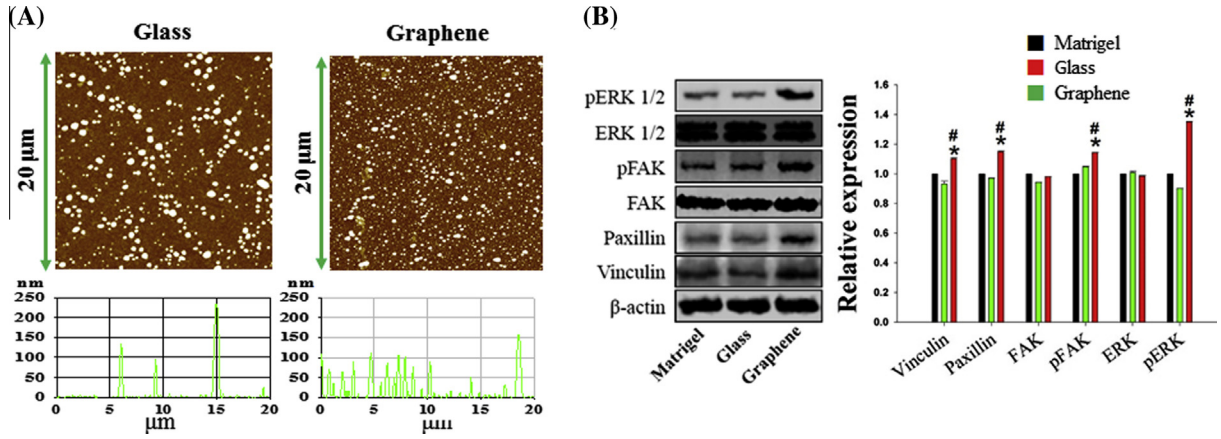

Fig. 4. Underlying mechanisms for the graphene-enhanced cardiomyogenic differentiation: Nanoroughness of graphene, FAK, and ERK signaling. (A) AFM images and surface nanoroughness of VN-coated glass (glass group) and VN-coated graphene (graphene group). (B) Western blot analyses of hESCs cultured on Matrigel-coated glass (Matrigel), VN-coated glass (glass), or VN-coated graphene (graphene) to analyze the molecules involved in the signal transduction pathways, and quantification of the relative protein expression levels of the cell signaling molecules. The values were normalized to the levels obtained for the Matrigel group ($n = 3$ per group, $^{*}p < 0.05$ versus Matrigel, $^{\#}p < 0.05$ versus glass).

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