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A Novel Approach via Surface Modification of Degradable Polymers With Adhesive DOPA-IGF-1 for Neural Tissue Engineering

Yi Zhang [1,2], Zongliang Wang [2], Yu Wang [2], Linlong Li [2], Zhenxu Wu [3], Yoshihiro Ito [4], Xiaoyu Yang [1,*], Peibiao Zhang [2,5,*]

[1] Department of Orthopaedics, The Second Hospital, Jilin University, Changchun 130041, PR China
[2] Key Laboratory of Polymer Ecomaterials, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, PR China
[3] School of Pharmaceutical Sciences, Jilin University, Changchun 130021, PR China
[4] Emergent Bioengineering Materials Research Team, RIKEN Center for Emergent Matter Science 2-1 Hirosawa, Wako-shi, Saitama 351-0198 Japan
[5] Institute of Applied Chemistry and Engineering, University of Science and Technology of China, Hefei 230026, PR China

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ABSTRACT

The highly damaging state of spinal cord injuries has provided much inspiration for the design of surface modification of the implants that can promote nerve regeneration and functional reconstruction. DOPA-IGF-1, a new recombinant protein designed in our previous study, exhibited strong binding affinity to titanium and significantly enhanced the growth of NIH3T3 cells on the surface of titanium with the same biological activity as IGF-1. In this article, surface modification of poly(lactide-co-glycolide) (PLGA) films with recombinant DOPA-IGF-1 was performed to promote the paracrine activity of human umbilical cord mesenchymal stem cells (hUCMSCs) by secreting neurotrophic factors. DOPA-IGF-1 exhibited the strongest binding ability to PLGA films than commercial IGF-1 and nonhydroxylated YKYKY-IGF-1. *In vitro* cultures of hUCMSCs on the modified PLGA films showed that DOPA-IGF-1@PLGA substrates significantly improved the proliferation, adhesion, and neurotrophic factors secretion of hUCMSCs, especially for nerve growth factor, as confirmed by qRT-PCR and western blot analysis. Subsequently, the acquired neurotrophic factors secreted by the hUCMSCs cultured on the DOPA-IGF-1@PLGA films obviously enhanced neurite outgrowth of PC12 cells. Taken together, PLGA substrates with DOPA-IGF-1 immobilization is a promising platform for neural tissue engineering via neurotrophic factors secretion from MSCs and should be further tested *in vivo*.

Introduction

Spinal cord injury (SCI) is a damaging state that causes permanent neurological deficits in injured persons.[1] As is well known, the limited regenerative capacity of central nervous system and the short of a physical matrix where neurons and endogenous repairing cells can adhere are 2 main reasons of poor recovery after SCI.[2] Consequently, biomaterials science and tissue engineering show great potential in new strategies for SCI treatment.

Nowadays, synthetic degradable polymers has gained much attention such as poly(lactide), poly(glicolide), and their copolymer poly(lactide-co-glycolide) (PLGA).[3] Their structure can be modulated to improve their biocompatibility by controlling their molecular weight and mole ratio of two monomers. PLGA is a kind of the most promising degradable materials in tissue engineering because the degradation rate of the copolymer can be adjusted by altering the ratio of lactic acid and glycolic acid.[4] Porous scaffolds,[5] microfibers,[6] conduits,[7] and films[8] based on PLGA matrix have been widely used in nerve regeneration and proved useful for SCI reconstruction *in vitro* and *in vivo*. At the same time, PLGA also shows great potential as a cell carrier in tissue regeneration.[9] However, hydrophobicity and lack of biofunctionality seriously restrict its biological applications. The surface modification of the PLGA implants toward improving biological performance has been a major focus of biomaterials research recently for developing functional nerve regeneration materials.

Several modification of existing synthetic degradable polymers approaches, such as grafting chains[10] or bioactive molecules[11] have been already widely used and these methods often alter the original material's physical properties. Matrix, growth factors, and cells are the three important elements in tissue engineering. Among them, growth factor proteins play an important role for tissue regeneration.[12] However, because of their instability and diffusible

* *Correspondence to:* Xiaoyu Yang (Telephone: +8613514460009) and Peibiao Zhang (Telephone: +8613578909239).
E-mail addresses: yangxiaoyu88@sina.com (X. Yang), zhangpb@ciac.ac.cn (P. Zhang).

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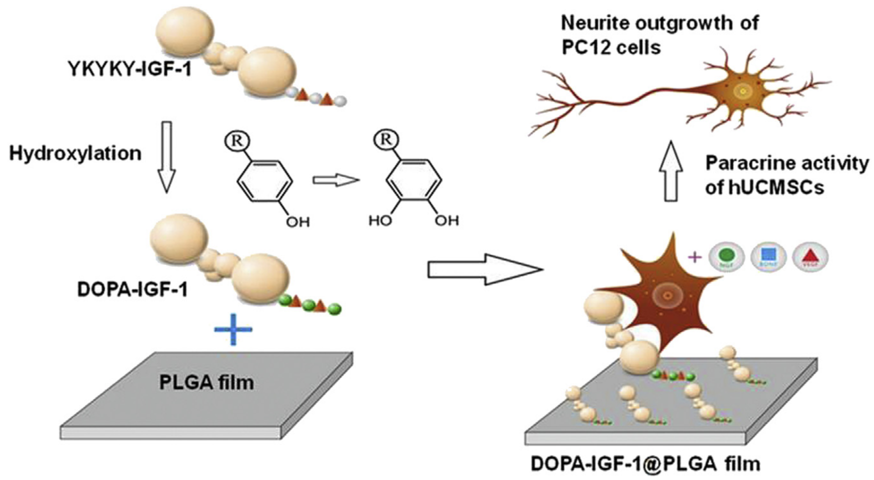

Scheme 1. Schematic illustration of DOPA-IGF-1 binding to a PLGA film and exhibiting significant bioactivity on neurite outgrowth of PC12 cells by promoting neurotrophic factor secretion of hUCMSCs. This DOPA-IGF-1@PLGA substrates–supported paracrine activity of MSCs may provide an attractive technology for neural regeneration applications.

nature when used *in vitro* and *in vivo*, many attempts have been made to improve the performance. To generate biofunctional substrates and regulate cell functions in a novel approach, conjugating growth factors and matrix has been performed by various researchers.[13] Sun et al.[14] produced a fusion protein using the collagen-binding nerve growth factor (NGF) combined with a collagen membrane for peripheral nerve regenaration. Another study reported fibrin-binding growth factor appeared to functionalize the fibrin in the form of improving wound-healing.[15] The adhesive protein secreted by marine mussels is widely used in many surface modifications of biomaterials.[16] Yang modified PLGA with DOPA to enhance contact guidance and neuronal differentiation of human neural stem cells.[17] In another study, DOPA was noted to coat the surface of TiO_2 nanoparticles to exhibit the proliferation of Schwann cells in peripheral nerve regeneration.[18] This indicated that DOPA-assisted immobilization resulted in large amount of peptides coated on the materials. It was confirmed that DOPA was a protein composed of a repeat sequence of DOPA and lysine. In our previous study, we introduced the site-specific DOPA into IGF-1 using recombinant DNA technique.[19] We incorporated Tyr-Lys-Tyr-Lys-Tyr (YKYKY) residues at the C-terminal of IGF-1, then hydroxylate the residues into DOPA-Lys-DOPA-Lys-DOPA (XKXKX, X = DOPA) via tyrosinase. When pH was regulated to 8.5, the oxidation reaction of catechol groups of DOPA was considered to resulted in chemical cross-linking; it demonstrated that this DOPA-IGF-1 would lead to the formation of a protein layer on the surface of titanium, enhanced the NIH3T3 adhesion, and increased the cell growth activity. It was noteworthy that as one kind of somatomedin, IGF-1 was produced by almost all types of neural cells in the brain.[20] It facilitates neuron progenitor proliferation, differentiation, and promotes cell survival through its antiapoptotic actions.[21] Furthermore, it has also been shown that IGF-1 could enhance mesenchymal stem cells (MSCs) pluripotency and self-renewal.[22] But few studies were reported about using DOPA-IGF-1 for biofunctionalization of implants in neural tissue regeneration. It was speculated that the binding affinity of this recombinant protein DOPA-IGF-1 to the surface of PLGA implants could be enhanced significantly by the hydroxylation of the tyrosine residues with tyrosinase.

Cell transplantation has been widely recognized for SCI. The studies of neural trans-lineage differentiation capability of MSCs have been reported, including astrocytes, oligodendrocytes, and even neurons.[23] However, this is still a controversial topic. The *in vivo* effects of MSCs are associated with their trophic activity via secretion of bioactive molecules and neurotrophic factors.[24] Human umbilical cord mesenchymal stems cells (hUCMSCs) are obtained painlessly from umbilical cord; thus fewer ethical issues and being hypoimmunogenic are two main advantages compared to other sources of MSCs.[25] Therefore, new strategies of using hUCMSCs for SCI have been recently developed on the regulation and improvement of their indirect role.[26]

Thus, in this study, binding growth factor DOPA-IGF-1 with a small pentapeptide tag composed of DOPA-Lys-DOPA-Lys-DOPA residues (XKXKX, X = DOPA) at its C-terminal was applied for the surface biofunctionalization of PLGA films using tyrosinase. The binding affinities of the DOPA-IGF-1 toward PLGA substrates were investigated through protein absorption, scanning electron microscopy (SEM), atomic force microscope (AFM), X-ray photoelectron spectroscopy (XPS) analysis, and hydrophilicity tests. The cell proliferation, adhesion, and neurotrophic factors secretion of hUCMSCs were investigated by culturing on the biomodified DOPA-IGF-1@PLGA substrates as shown in Scheme 1. The aim of this study was to explore the effectiveness of biofunctionality with the binding proteins of DOPA-IGF-1 and provide a novel surface modification method of PLGA implants for neural tissue engineering.

Materials and Methods

Preparation of PLGA Films

PLGA (lactide/glycolide ratio = 80/20, Mn = 85,000), synthesized in our laboratory according to the literature procedure,[27] was dissolved in chloroform to obtain a 3% (w/v) PLGA/chloroform solution. Then the solution was spread on an 8-mm cover slide as a 0.25-mm thick layer preliminary treated with 2% dimethyldichlorosilane (DMDC; Fluka). The PLGA-coated slides were vacuum-dried for 48h under ambient temperature for the further use.

Preparation of Y-IGF-1

The expression and purification of recombinant YKYKY-IGF-1 (Y-IGF-1) were performed as literature reported previously.[19] In brief, the recombinant pET15-Y-IGF-1 was transformed into the BL21 (DE3) strain of *Escherichia coli*. The bacterial culture containing the recombinant pET15-Y-IGF-1 expression vector was inoculated in 100 mL lysogeny broth medium containing 100 μg/mL ampicillin in a 500-mL flask. The mixture were grown overnight at 37°C with shaking and then transferred to a 2-L flask containing 1 L of sterilized terrific broth medium and 100 μM ampicillin at 37°C with vigorous shaking until the optical density$_{600}$ was 0.8. Isopropyl-β-d-thiogalactoside was added for expression to a final concentration of 0.1 mM with overnight growth. Recombinant proteins from *E coli* were purified as described in the manual (Qiagen). The cleared lysate supernatant was purified over a Ni-NTA column. Nonobjective proteins were removed by washing the column with wash buffer. Y-IGF-1 was eluted from the Ni-NTA spin column with elution buffer. Then Y-IGF-1 protein was dialyzed with PBS for removing urea in a semipermeable membrane. After dialysis, Y-IGF-1 protein solution was put into a 50-mL centrifuge tube, frozen at −80°C for 30 min, and freeze-dried to obtain protein powder.

Tyrosine Hydroxylation

The Y-IGF-1 protein was diluted with PBS to 1 mg/mL. The tyrosine residues of Y-IGF-1 were hydroxylated using solutions of tyrosine hydroxylase (500 μg/mL, 500 μL) (Sigma-Aldrich, St. Louis, MO), ascorbic acid (250 mM, 500 μL), Y-IGF-1 (1 mg/mL, 500 μL), and PBS (500 μL, pH 7.2) at room temperature for 2 h, then regulated PH to 8.5.

SDS-PAGE and Western Blot Assays

SDS-PAGE and western blot assays were performed using an established protocol.[28] SDS-PAGE analysis was performed using a 15% gel for different protein fractions according to the manual. For western blot, migrated protein band in the gel were transferred to a polyvinylidene difluoride membrane (Roche Diagnostic, Boston, MA) using an electroblotting apparatus (Bio-Rad) at 200 mA for 40 min in 25 mM Tris−192mM glycine. The membrane was blocked for 1 h by incubating with 10% bovine serum albumin in Tris-HCl-buffered saline and Tween 20 (TBST) solutions, and then incubated with anti-IGF-1 polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). After washing, the membrane was incubated with anti-IgG (Santa Cruz Biotechnology Inc.) added at a dilution of 1:1000 in TBST solution. The bound antibody was detected using enhanced chemiluminescence.

Protein Adsorption on the PLGA Films

The obtained DOPA-IGF-1, Y-IGF-1, and commercial IGF-1 (cIGF-1) were prepared as a solution of 500 ng/mL in PBS. The PLGA films were immersed in the DOPA-IGF-1, Y-IGF-1, and cIGF-1 protein solutions for up to 12 h at 4°C while shaking (60 rpm) to enable homogeneous dispersion of protein. The amount of DOPA-IGF-1, Y-IGF-1, and cIGF-1 proteins adsorbed on the PLGA films were determined through the decrease of the proteins within the solutions using double-antibody sandwich ELISA method (ab108873; Abcam, Cambridge, UK). All the samples were 500 times diluted for detection to fit the sensitivity of ELISA assay (2.74-2000 pg/mL). It was determined through HRP-conjugated secondary antibody (1:500; Bioss, Shanghai, China) in Turbo-ELISA substrate solution (Thermo Scientific) at an absorbance 450 nm using an ELISA plate reader.

Surface Morphology Analysis and Surface Characterization

The morphology of aforementioned DOPA-IGF-1@PLGA, Y-IGF-1@PLGA, cIGF-1@PLGA, and pure PLGA films were observed by SEM (Zeiss supra 40 VP scanning electron microscope) and AFM (multimode scanning probe atomic force microscope, Veeco Instruments). The surface composition of different samples was detected using XPS (Thermo) to obtain the elemental compositions.

Table1
Primers Used in Quantitative Real-Time Polymerase Chain Reaction for hUCMSCs

Gene	Forward Primer Sequence (5′−3′)	Reverse Primer Sequence (5′−3′)
NGF	AGCGCAGCGAGTTTTGG	AGAAAGCTGCTCCCTTGGTAA
BDNF	GCCAGAAAAAGCCAAGGAGT	ATGCCTCCCATATGCAGACT
VEGF	ATCTGCATGGTGATGTTGGA	GGGCAGAATCATCACGAAG
GAPDH	GCTCCCTCTTTCTTTGCAGC	ACCATGAGTCCTTCCACGAT

In addition, the water contact angle for all the different substrates was measured on a Krüss DSA 10 instrument.

In Vitro *Culture of hUCMSCs Cells*

The hUCMSCs were obtained from Jilin Heze Biotechnology Co., Ltd. and cultured in high glucose DMEM medium (HyClone) supplemented with 10% fetal bovine serum (Invitrogen), penicillin G (100 units/mL), streptomycin (100 μg/mL), and amphotericin B (25 mg/mL).[29] The cells were incubated at 37°C in the incubator with 5% CO_2 and 95% humidity. The hUCMSCs were cultured to 80% confluency and then passaged for expansion. hUCMSCs of passages 2 or 3 were used for the experiments.

Cell Proliferation and Adhesion

The DOPA-IGF-1@PLGA, Y-IGF-1@PLGA, and cIGF-1@PLGA films were washed with PBS and placed into a 24-well plate. PLGA films immersed in PBS at the same conditions were used as control. hUCMSCs cells were seeded on different groups at initial densities of 20,000 cells per well. After 1, 3, and 7 days of culture, cell proliferation was studied by Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). After 2 h of incubation with CCK-8 solution added into the wells, 200 μL of the reaction solution was transferred into a new 96-well plate, and the absorbance values at 450 and 600 nm were measured on a multifunctional microplate scanner (Tecan Infinite M200). For morphological description of cell adhesion at 24 h and 72 h, actin filaments of cells were stained with Acti-stain 555 phalloidin, and the cell nucleus was stained with DAPI.

Paracrine Activity of hUCMSCs

The secretion of neurotrophic factors from hUCMSCs cultured for 7 days and 14 days on the different PLGA films was assessed. The mRNA levels of representative neurotrophic factors including NGF, brain-derived neurotrophic factor (BDNF), and vascular endothelial growth factor (VEGF) were detected by quantitative real-time polymerase chain reaction (qRT-PCR). After culture for different time intervals, cells were gathered from each sample and total RNA was isolated using the TRIzol Reagent (Invitrogen), according to the manufacturer's protocol. The RNA was reverse-transcribed to cDNA using PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time, TaKaRa). PCR amplification was performed using qPCR SYBR Green Mix Kit (TaKaRa). The relative transcript quantities were calculated using the cycle threshold method with the GAPDH as the endogenous reference gene amplified from the samples. The primers of genes are shown in Table 1.

Table 2
Primers Used in qRT-PCR for PC12 Cells

Gene	Forward Primer Sequence (5′−3′)	Reverse Primer Sequence (5′−3′)
SYN1	TCCTCCTGCTCAACAACGAC	GGAGGGGCTGGCTTTGAG
MAP2	CCAGAAGTCCCCATGGCAAA	GCTGGTGGTATGTTCTGGCT
GAPDH	GCATCTTCTTGTGCAGTGCC	TACGGCCAAATCCGTTCACA

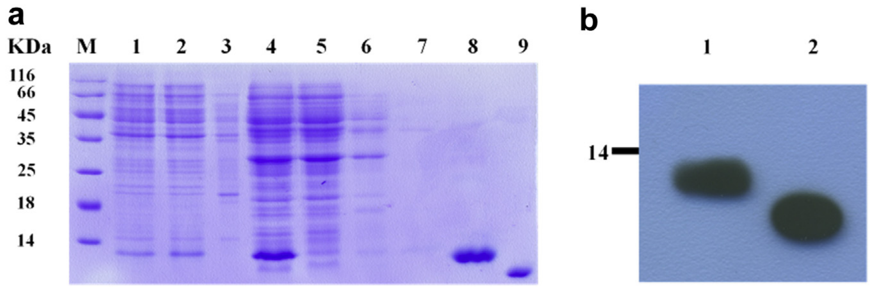

Figure 1. SDS-PAGE and western blot of Y-IGF-1 fusion protein. (a) SDS-PAGE analysis of purified fusion proteins. The values shown on the left of the figure are the measured molecular weights. M, marker; 1, before induction; 2, after induction; 3, supernatant; 4, inclusion body; 5–7, flows; 8, Y-IGF-1; 9, cIGF-1. (b) Western blot of purified Y-IGF-1 (1) and cIGF-1 (2) proteins analyzed with mouse anti-IGF-1 and HRP-labeled rabbit anti-mouse IgG antibody.

Based on the gene expressions of neurotrophic factors, NGF was chosen for further analysis of protein levels. The NGF protein secreted by hUCMSCs in the medium was analyzed by western blot with anti-NGF antibody. Culture medium of different films were harvested and 50 μL of the medium was boiled with 10 μL 5× sample buffer (60 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 5% b-mercaptoethanol, and 0.01% bromophenol blue) for 10 min. 10 μL of each boiled sample was loaded to wells of a gradient gel (Bio-Rad, Hercules, CA), then the proteins were transblotted to a nitrocellulose membrane at ~2.5 h at 350 mA. The transblotted nitrocellulose membrane was blocked by nonfat powdered milk TBST (Tris buffer with 0.5% Tween-20) for 2 h while shaking (60 rpm) at room temperature. Primary antibody (mouse anti-NGF antibody; Santa Cruz Biotechnology) was diluted at 1:1000 in the nonfat powdered milk TBST solution and incubated with the membrane overnight in a 4°C chamber. After washing by 0.5% TBST, the membrane was incubated with rabbit anti-mouse IgG conjugated to HRP (ABCAM) diluted 1:2000. The bound antibody was detected using enhanced chemiluminescence.

PC12 Cells Morphology Assay to Confirm the Secretion of Neurotrophic Factors

To confirm the secretion of neurotrophic factors by hUCMSCs cultured on the different PLGA films, all the mediums were collected referred to as conditioned mediums (CMs) from these cell cultures, respectively. PC12 cells were used to investigate the effects of secreted neurotrophic factors, particularly NGF from CMs. It was because PC12 cells are well-known to be highly responsive to NGF in the neuritogenesis and differentiation.[30] Cells were maintained in RPMI 1640 (ATCC Cell Biology, Manassas, VA) containing 10% heat-inactivated equine serum (SH30074; Hyclone, South Logan, UT) and 5% fetal bovine serum, at 37°C in the incubator with 5% CO_2 and 95% humidity. To observe neurite outgrowth, PC12 cells were plated at 10,000 cells per well of a 24 well plate. The CMs obtained from hUCMSCs cultured on the PLGA, cIGF-1@PLGA, Y-IGF-1@PLGA, and DOPA-IGF-1@PLGA films were centrifuged at 1000 rpm for 5 min to remove the suspended hUCMSCs. PC12 cells were grown in the different CMs. PC12 cells which were maintained in maintenance medium (MM) were set as control. The neurite outgrowth patterns of PC12 cells were observed by optical microscopy at 1, 3, and 6 days. Based on the optical microscopy photos, neurite outgrowth patterns, such as number of cells with neurite outgrowth and average length of neurite outgrowth, were analyzed.

Neurogenic Genes Expression

PC12 cells were incubated in CMs or MM for 3 days and 6 days and the expression of neurogenic genes was explored via qRT-PCR. The extraction of RNA and analysis by qRT-PCR was conducted according to the procedure as mentioned previously. Specific PCR primer sequences are given in Table 2.

Statistical Analysis

The data were presented as the mean values ± standard deviations. Statistical analyses were performed using Origin 8.0 for paired samples and ANOVA for multiple samples. A value of $p < 0.05$ was considered statistically significant.

Results and Discussion

Preparation of DOPA-IGF-1

After induction with 0.2 mM isopropyl-β-d-thiogalactoside at 22°C, the BL21 (DE3) *E coli* containing pET15-Y-IGF-1 produced a clear 10.5 kDa band detected by SDS-PAGE as shown in Figure 1a. The result showed that recombinant Y-IGF-1 protein could be expressed efficiently in *E coli*. Then Y-IGF-1 was purified and refolded by affinity chromatography and desalting chromatography. Western blot was performed for purified Y-IGF-1 and cIGF-1 using the mouse anti-human IGF-1 monoclonal antibody and rabbit anti-mouse IgG conjugated to HRP. As shown in Figure 1b, we observed a 10.5 kDa band for Y-IGF-1 and a 7.4 kDa band for cIGF-1, which was in accordance with the result of SDS-PAGE (Fig. 1a). These results demonstrated that Y-IGF-1 could bind to IGF-1 antibody specifically, and these bands were correspondence with the theoretical molecular weights. These data indicated that the recombinant Y-IGF-1 presented in the *E coli* cell supernatants and it

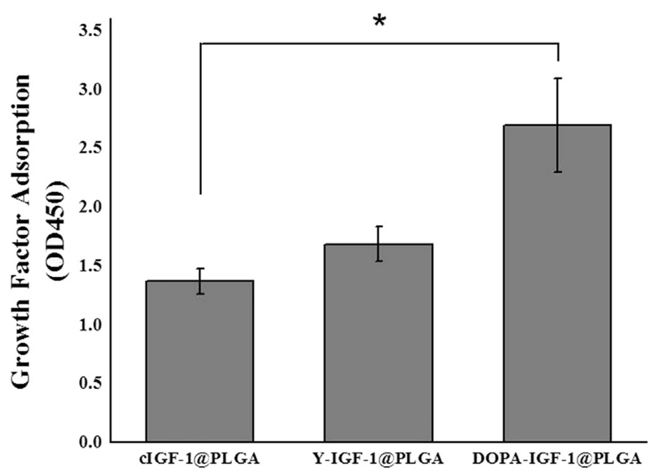

Figure 2. The adsorption ability of DOPA-IGF-1, Y-IGF-1, and cIGF-1 binding to the PLGA films. Asterisks (*) indicate statistical significance, $p < 0.05$.

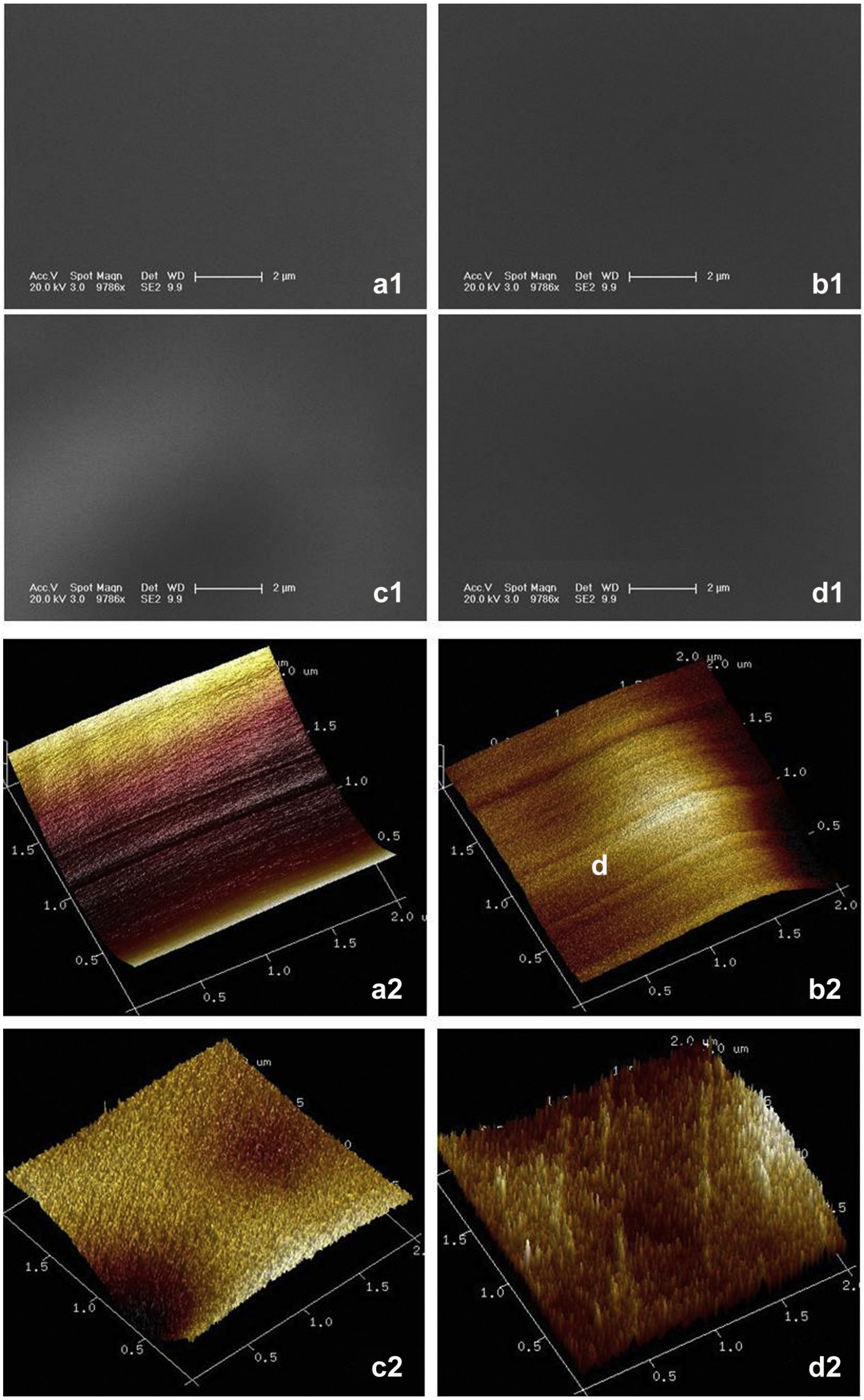

Figure 3. SEM and AFM images of the surface of different PLGA films. (a) PLGA, (b) CIGF-1@PLGA, (c) Y-IGF-1@PLGA, (d) DOPA-IGF-1@PLGA. The scale bars for SEM are 2 μm, the scale scise for AFM is 2 μm × 2 μm.

could be isolated with a high degree of purity by affinity chromatography on nickel-chelating sepharose.

Neurotrophic factor supplementation plays important roles in the regeneration of central nerve injury, which can stimulate axons regeneration, regulate inflammatory cells, protect the injured neurons, and inhibit scar formation.[31] Growth factors also promote MSCs proliferation, differentiation, and maturation.[32] However, because of their instability and diffusible manner, many attempts have been made to improve the performance of growth factors.[12] Since recombinant DNA technology arose, designs of binding growth factors with target-binding peptides have been developed. These recombinant growth factors not only bind to biological substrates, but also to artificial substrates. The DOPA-assisted immobilization strategy was inspired by mussel-adhesion phenomena in nature.[33] Ko and Yang reported that PLGA films were more rougher and wetter after coated with DOPA.[34] Considering DOPA is a noncanonical amino acid, YKYKY was firstly incorporated into IGF-1 at the terminus and subsequently converted these residues into DOPA using a tyrosinase.

Adsorption Rate of Protein on PLGA Films

The DOPA-IGF-1 protein was obtained after hydroxylation using tyrosine hydroxylase. The protein adsorption studies of DOPA-IGF-1, Y-IGF-1, and cIGF-1 onto PLGA films are shown in Figure 2. After incubation for 12 h, it could be seen that the DOPA-IGF-1 and Y-IGF-1 adsorption on PLGA films was obviously higher than those of cIGF-1 adsorption on PLGA films. CIGF-1 showed the lowest protein adsorption capacity on PLGA films for its weak interactions on the surface of PLGA. Moreover, YKYKY residues were converted into DOPA-Lys-DOPA-Lys-DOPA (XKXKX) after tyrosine hydroxylation, the DOPA-IGF-1 adsorption capacities on PLGA films were the highest in comparison with Y-IGF-1 on PLGA films. The adsorption capacity of Y-IGF-1 was also higher than cIGF-1 because of YKYKY-contained lysine. Previous studies illustrated lysine molecules adsorb strongly to various solid surfaces, leaving cationic sites which combine with the anionic sites on cell surface.[35] More DOPA-IGF-1 was absorbed than Y-IGF-1 and cIGF-1 because DOPA-IGF-1 contains DOPA. Nowadays, DOPA-promoted surface functionalization of materials had been used for tissue engineering.[33] In our previous studies, DOPA deposition processes for incorporation of peptides[36] or growth factors[37] on scaffolds or microcarriers that efficiently regulate osteogenic differentiation of preosteoblasts of cells. In this study, when pH was regulated to 8.5, the YKYKY residues were hydroxylated into DOPA-Lys-DOPA-Lys-DOPA via tyrosinase, the catechol groups of DOPA oxidized to form a protein layer (DOPA-IGF-1) on the surface of a wide range of organic materials, such as polymers.[38] Meanwhile, previous studies reported binding IGF-1 on the surface of materials. For example, Van Lonkhuyzen et al. produced a chimeric protein of IGF-I and vitronectin (VN),[39] and the VN: IGF-I/IGF-BP complex exhibited a higher cell proliferation activity via receptor-mediated and integrin-mediated pathways. In our study, the binding ability of DOPA-IGF-1 was higher than Y-IGF-1 and cIGF-1 on the PLGA films. It indicated that it regulated cellular functions more efficiently at the same concentration.

Surface Morphology

The surface morphology of the materials, such as surface topology (roughness), greatly affect the cell adhesion, spreading, and proliferation.[40] Figure 3 displays the topology and roughness of bare and DOPA-IGF-1–, Y-IGF-1–, and cIGF-1–modified PLGA films analyzed by SEM and AFM. No morphological difference was observed from the SEM images, and all the PLGA films exhibited smooth surfaces before and after surface modification. Subsequently, further analysis of morphology and topology of different PLGA films was performed via AFM. The pure PLGA and cIGF-1@PLGA films exhibited regular and smooth surface with surface roughness root mean square (RMS) 0.529 nm and 0.543 nm via analyzing the topography scans of the film's surface. This might be due to the weak interaction between cIGF-1 and PLGA. However, Y-

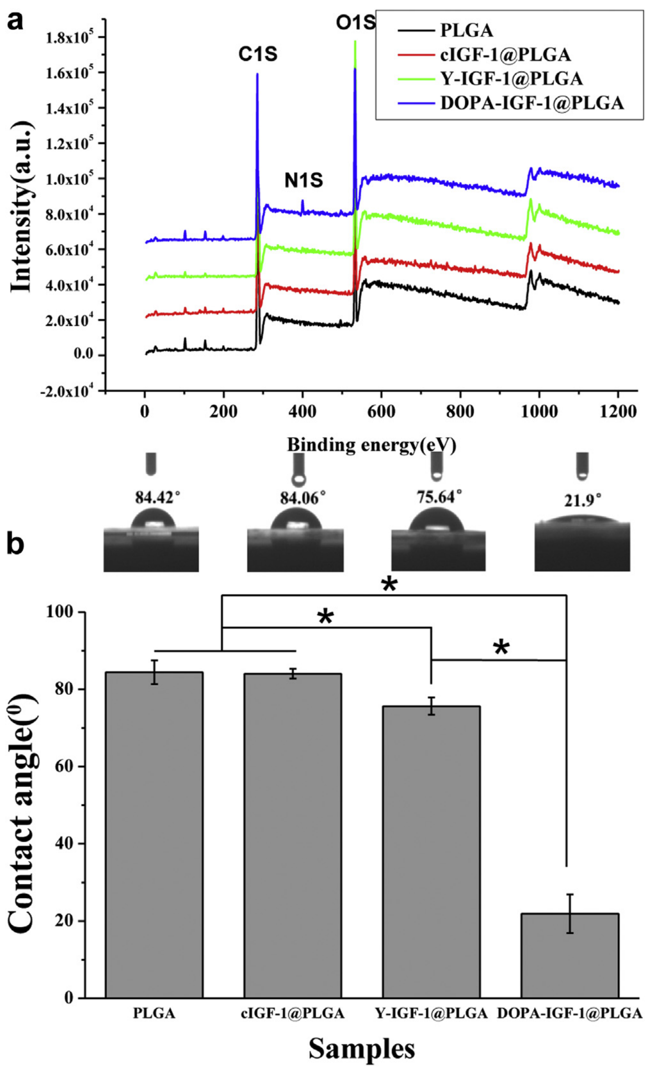

Figure 4. Surface characterization of DOPA-IGF-1–, Y-IGF-1–, and cIGF-1–modified PLGA films. (a) XPS analysis of different samples. (b) Water contact angle measurement for different samples. Asterisks (*) indicate statistical significance, $p < 0.05$.

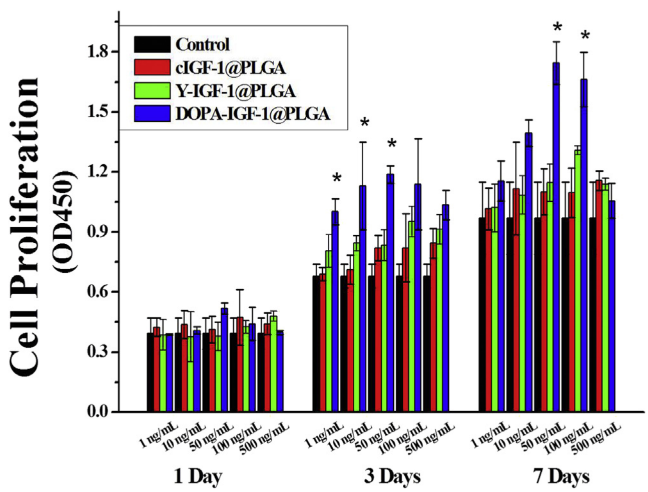

Figure 5. Proliferation of hUCMSCs for 1, 3, and 7 days on PLGA, cIGF-1@PLGA, Y-IGF-1@PLGA, and DOPA-IGF-1@PLGA films, and the concentrations of cIGF-1, Y-IGF-1, and DOPA-IGF-1 solutions for the protein immobilization were 1, 10, 50, 100, and 500 ng/mL, respectively. Asterisks (*) indicate statistical significance, $p < 0.05$.

IGF-1@PLGA demonstrated rough and irregular surface morphology with surface roughness RMS 0.764 nm due to the existence of lysine. While there was more rugged surface morphology in DOPA-IGF-1@PLGA group and its surface roughness RMS was 1.39 nm. Ellipsoidal particles were observed on the DOPA-IGF-1—modified films at protein concentrations of 500 ng/mL, as shown in Figure 3d2. These results demonstrated that surface functionalization of PLGA with DOPA-IGF-1 could enhance the surface topology, which may facilitate initial cellularization.

Surface Characterization of DOPA-IGF-1—, Y-IGF-1—, and cIGF-1—Modified PLGA Films

After the surface modification of DOPA-IGF-1, Y-IGF-1, and cIGF-1, the XPS analysis of PLGA films was shown in Figure 4a. After immersed in 500 ng/mL of protein solutions at 4°C for 12 h, for DOPA-IGF-1@PLGA films, a very high nitrogen peak (N1s) additionally appeared at ~400 eV, which indicated successful immobilization of protein on the substrates. This was due to the existence of DOPA-binding domain. In contrast to DOPA-IGF-1@PLGA films, Y-IGF-1@PLGA films exhibited a small nitrogen peak (N1s). However, there was no nitrogen peak (N1s) for cIGF-1@PLGA and PLGA films. Probably, the physically adsorbed IGF-1 was diffusible and unstable. This hypothesis was verified in the following cell experiments.

The hydrophilicity of materials plays an important role in interacting with cells.[41] We measured the water contact angle of PLGA, cIGF-1@PLGA, Y-IGF-1@PLGA, and DOPA-IGF-1@PLGA films to analyze the change in wetting of the PLGA surface. As shown in Figure 4b, the contact angle on pure PLGA films was 84.06 ± 3.1°, and was 84.42 ± 1.26° for cIGF-1@PLGA films. It indicated that the wettability of PLGA substrates was not improved by the physically adsorbed cIGF-1 in 500 ng/mL protein solutions. The water contact angle for Y-IGF-1@PLGA samples was decreased compared with those of the bare PLGA and cIGF-1@PLGA samples, with a statistically significant difference ($p < 0.05$). After binding to DOPA-IGF-1, the average water contact angle dramatically decreased from 75.64 ± 2.26° for the Y-IGF-1@PLGA films to 21.9 ± 5.0°, indicating the maximum wettability attributed to DOPA-IGF-1 immobilization. These results were correlated with the AFM, which demonstrated that surface topology and hydrophilicity of DOPA-IGF-1@PLGA films were both highly increased via efficient immobilization.

Cell Proliferation and Morphology on DOPA-IGF-1@PLGA Films

The enhancement of cell proliferation and adhesion on the nerve scaffolds is usually responsible for functional recovery of the injured spinal cord.[42] The proliferation of hUCMSCs was assessed using CCK-8 assay from 1 to 7 days and shown in Figure 5. The cell proliferation was observed to rise with the increase of cIGF-1@PLGA, Y-IGF-1@PLGA, and DOPA-IGF-1@PLGA films. IGF-1 has been shown to enhance proliferation of MSCs *in vitro*.[43] Similar results were also found in this study. The optical density values of cell viability were slightly increased for the cIGF-1@PLGA compared with pure PLGA films, but there was no statistical significance ($p > 0.05$). In addition, the bioactivity of Y-IGF-1@PLGA and DOPA-IGF-1@PLGA films markedly increased at 10 ng/mL and 50 ng/mL ($p < 0.05$) at 3 and 7 days. It was concluded that 50 ng/mL DOPA-IGF-1 was the most effective in the enhancement of cell proliferation among the five concentrations used in this study. However, the promotion of cell proliferation seemed to be decreased when the density of DOPA-IGF-1 solution was 100 ng/mL and Y-IGF-1 was 500 ng/mL. Cell proliferation was significantly suppressed at 500 ng/mL of DOPA-IGF-1. It indicated that there was dose correlation between IGF-1 and cell viability and small dose (not more than 100 ng/mL) of DOPA-IGF-1 played a better biological effect owing to its inherent adhesion. In this study, hUCMSCs proliferation was greatly promoted by 50 ng/mL of DOPA-IGF-1 immobilized PLGA films. So the concentration of 50 ng/mL was applied in the subsequent experiments.

As shown in Figure 6, the adhesion of the hUCMSCs grown on PLGA films at 24 and 72 h was observed using confocal laser

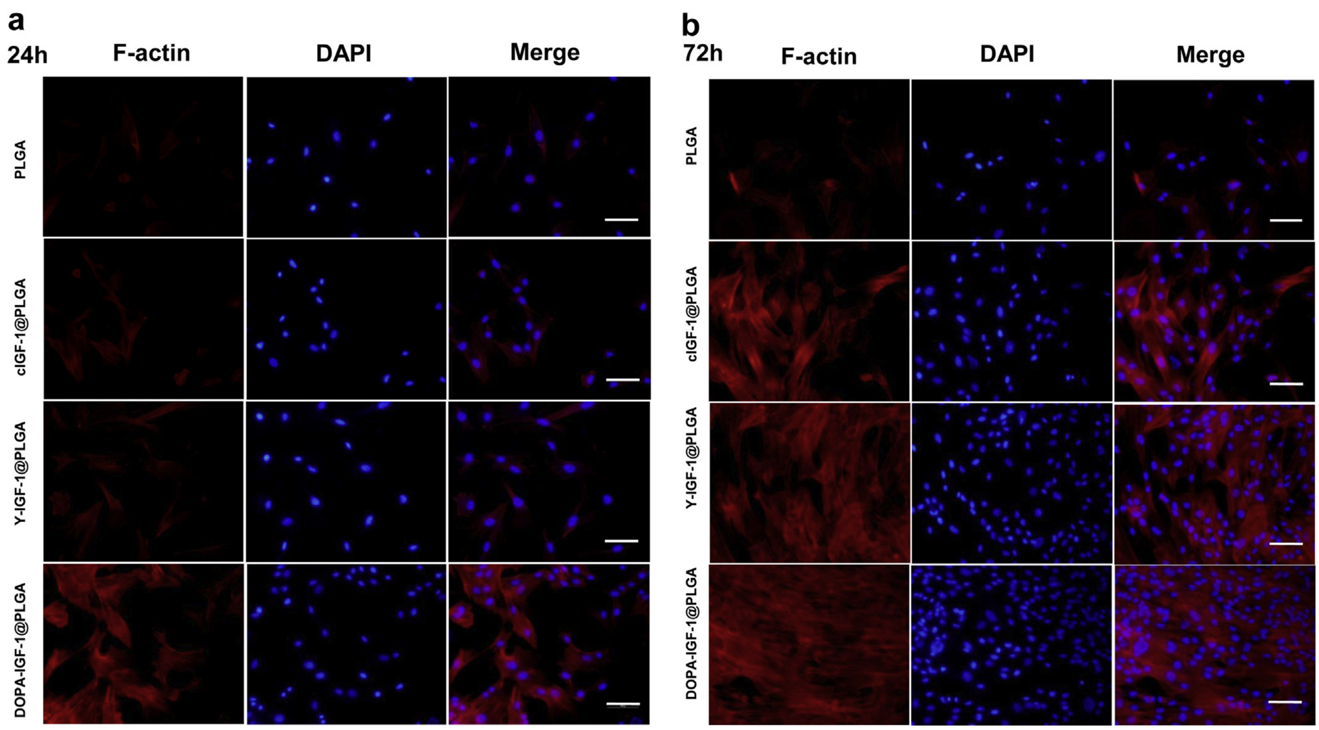

Figure 6. (a and b) Cell morphology of hUCMSCs grown on the PLGA, cIGF-1@PLGA, Y-IGF-1@PLGA, and DOPA-IGF-1@PLGA films were assayed by confocal laser scanning microscope. The actin filaments (red) were stained by phalloidin and the cell nucleus (blue) was stained by DAPI. Scale bars are 100 μm.

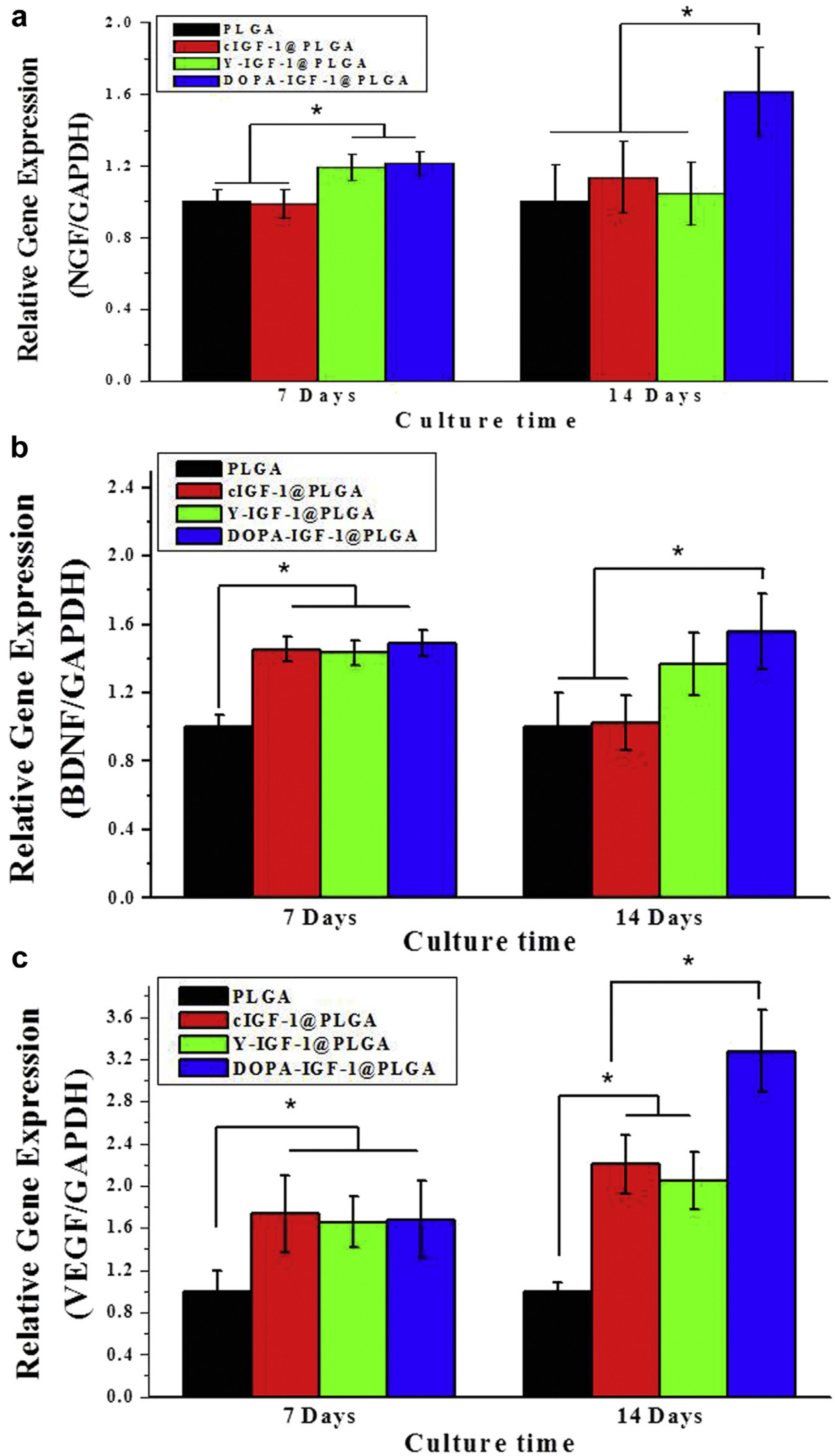

Figure 7. Neurotrophic factors expressed by the hUCMSCs cultured on different substrates assessed by the mRNA levels for 7 and 14 days. The expression of three neurotrophic factors genes (a) NGF, (b) BDNF, (c) VEGF) was analyzed by qRT-PCR. Asterisks (*) indicate statistical significance, $p < 0.05$.

scanning microscope by actin microfilaments (red) and nuclei (blue) staining. The attachment of cells cultured on Y-IGF-1@PLGA and DOPA-IGF-1@PLGA samples was better compared with that on bare PLGA and cIGF-1@PLGA samples due to the surface topology and hydrophilic enhancement on lysine residues and immobilization of DOPA residues, respectively. Furthermore, there were greater cell quantities and positive cellular interactions on DOPA-IGF-1–immobilized films. This might be due to efficient immobilization of DOPA-IGF-1. In addition, the cell adhesion on the cIGF-1@PLGA films was also better than that of the pure PLGA samples at any time point, which likely contributed to the physically adsorbed cIGF-1, but the cell adhesion was still unsatisfactory.

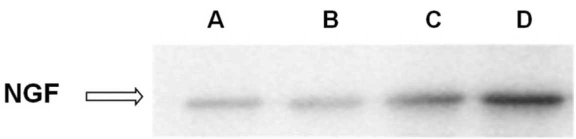

Figure 8. Protein level of neurotrophic factors (NGF) assessed by western blot analysis after 14 days of culture on different substrates of (a) PLGA, (b) cIGF-1@PLGA, (c) Y-IGF-1@PLGA, and (c) DOPA-IGF-1@PLGA.

Paracrine Activity of hUCMSCs Grown on Different PLGA Films

hUCMSCs are capable of secreting certain neurotrophic factors, such as NGF, BDNF, and VEGF during SCI for enhancing regenerating axon growth and neuron survival.[44] Therefore, the degree of secreted neurotrophic factors can be considered another measure of cell differentiation.[45] As such, NGF, BDNF, and VEGF expressed by hUCMSCs cultured within different PLGA films for 7 and 14 days were assessed by qRT-PCR (Fig. 7). The expression of NGF in the Y-IGF-1@PLGA and DOPA-IGF-1@PLGA groups was higher than that of the other two groups at 7 days ($p < 0.05$). At 14 days, the expression was much stronger for the DOPA-IGF-1@PLGA groups compared with other samples ($p < 0.05$, Fig. 7a). The levels of BDNF and VEGF gene expression for cIGF-1@PLGA, Y-IGF-1@PLGA, and DOPA-IGF-1@PLGA were slightly higher than pure PLGA samples ($p < 0.05$) at 7 days. Furthermore, there were no significant differences on the cIGF-1@PLGA, Y-IGF-1@PLGA, and DOPA-IGF-1@PLGA groups ($p > 0.05$, Figs. 7b and 7c). At 14 days, the BDNF gene expression was higher in DOPA-IGF-1@PLGA groups than cIGF-1@PLGA and PLGA samples ($p < 0.05$, Fig. 7b). The expression of VEGF was stronger for the DOPA-IGF-1@PLGA groups compared with other samples ($p < 0.05$, Fig. 7c).

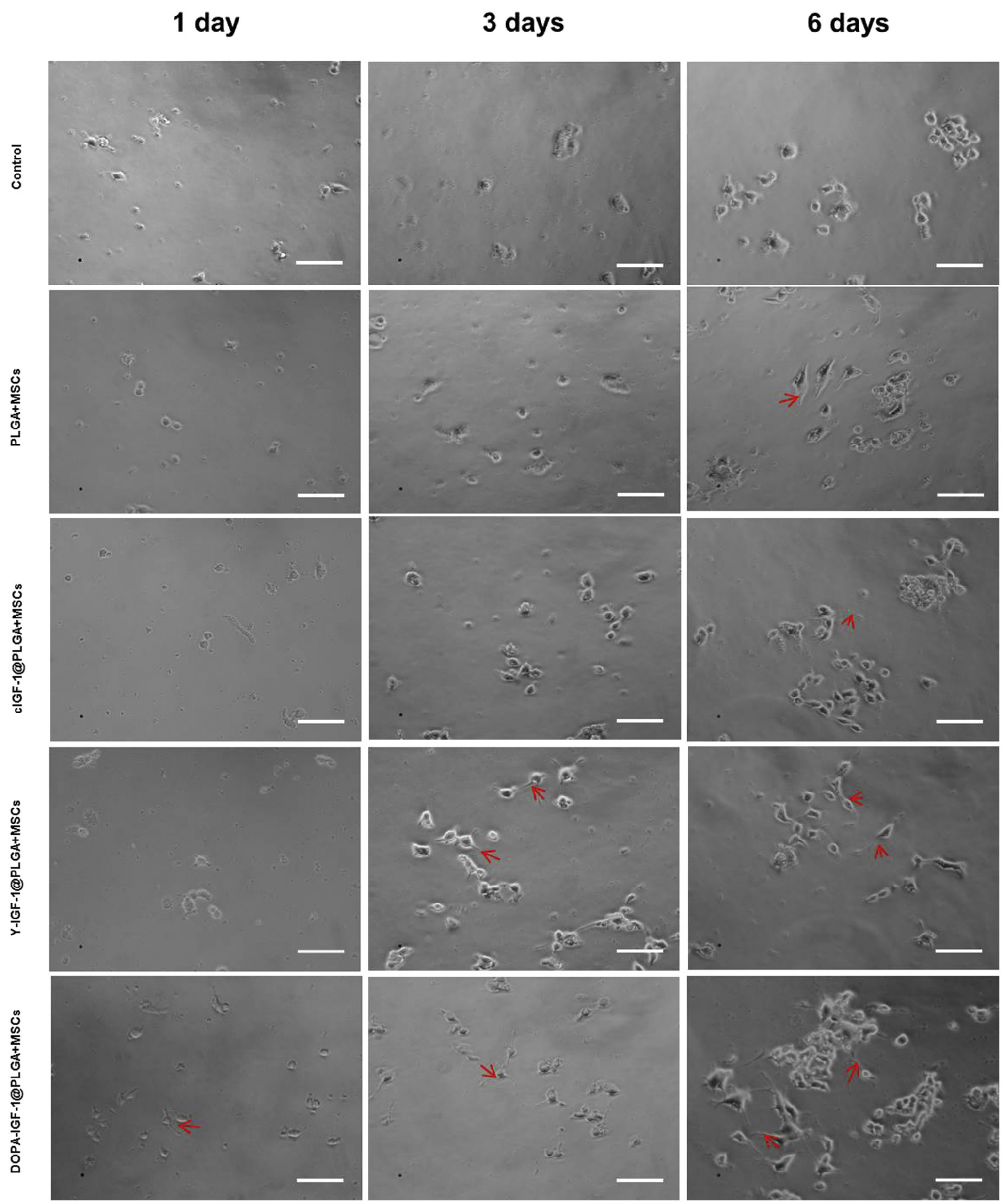

Figure 9. The neuritogenesis behavior of PC12 cells was influenced by the release of neurotrophic factors (especially for NGF) from the hUCMSCs cultured on different samples for 1, 3, and 6 days. Red arrows were used to mark the axons. Scale bars are 100 μm.

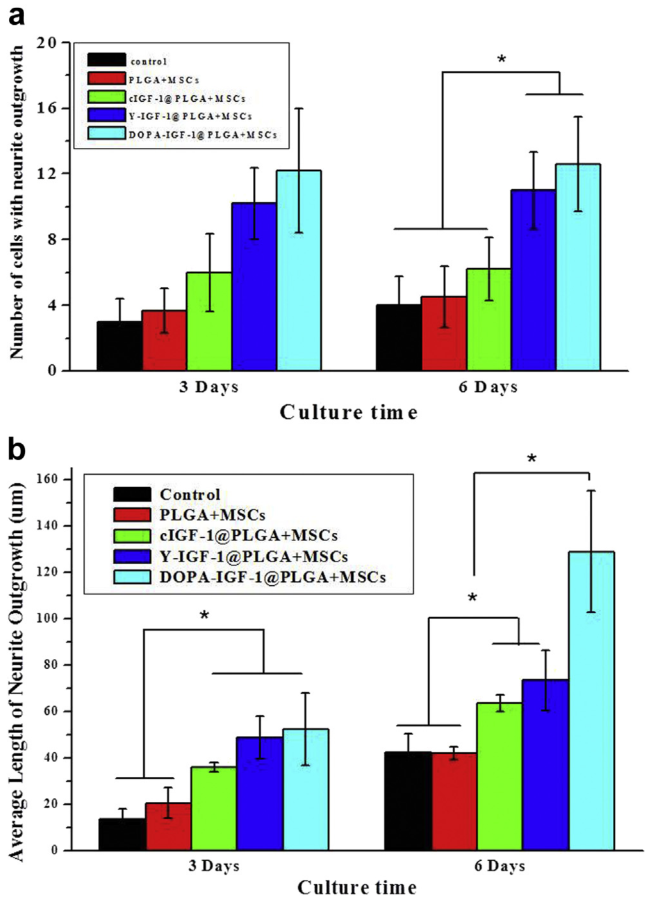

Figure 10. Effects of neurite outgrowth of PC12 cells cultured on the different samples. Parameters including (a) number of cells with neurite outgrowing and (b) average length of neurite outgrowth were shown for 3 and 6 days. Asterisks (*) indicate statistical significance, $p < 0.05$.

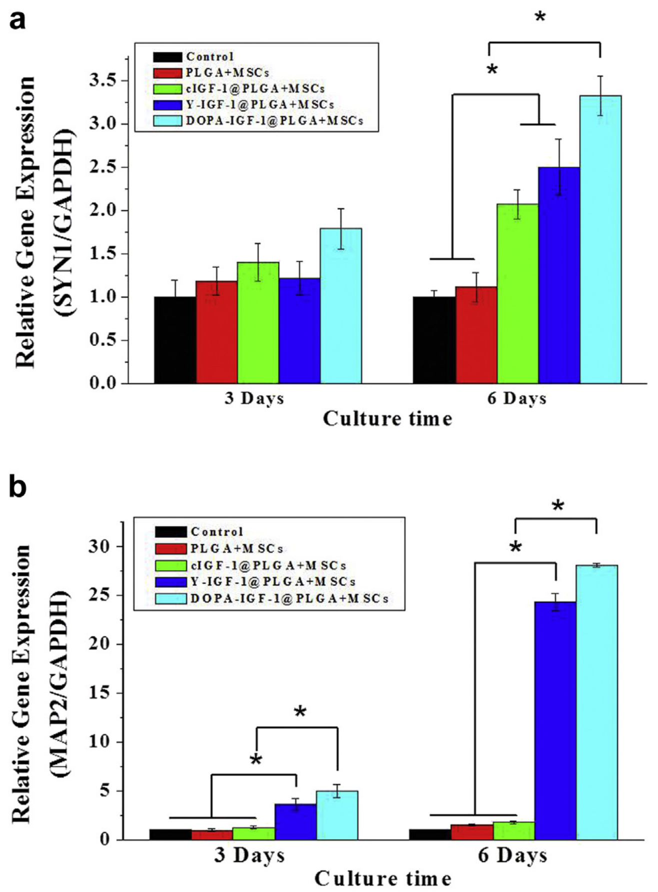

Figure 11. PC12 cells were cultured on the different samples for 3 and 6 days. SYN1 (a) and MAP2 (b) genes were significantly up-regulated for the group of DOPA-IGF-1@PLGA + MSCs. Asterisks (*) indicate statistical significance, $p < 0.05$.

The protein levels of NGF was further assessed by gathering the supernatants of culture medium at 14 days and analyzed by western blot (Fig. 8). There was a weakest protein band signal for PLGA films. However, the NGF level from cIGF-1@PLGA samples was not found to be significantly different with PLGA. Furthermore, the band expressions were more evident for the Y-IGF-1@PLGA and DOPA-IGF-1@PLGA, particularly the DOPA-IGF-1@PLGA samples.

It is indicated that IGF-1 plays an important role in hUCMSCs differentiation toward neurons, astrocytes, and neural progenitor cells (NPCs).[46] However, strategies to use paracrine activity of hUCMSCs in SCI have been paid more and more attention. In this case, IGF-1 receptor can induce transcriptional activity to promote survival, differentiation, and paracrine activity of hUCMSCs.[47] We investigated the expression of a series of genes including NGF, BDNF, and VEGF. The mRNA levels, particularly those for NGF and VEGF, were expressed significantly higher in the DOPA-IGF-1@PLGA groups at 14 days. Compared with these results, the expression of NGF for the DOPA-IGF-1@PLGA samples was not obviously higher than that of Y-IGF-1@PLGA at 7 days, even though they both expressed stronger than the pure PLGA and cIGF-1@PLGA groups. This was because YKYKY residues contained lysine which could adsorb strongly to various solid surfaces and leave cationic sites which combine with the anionic sites on cell surfaces before tyrosine hydroxylation. After hydroxylation, YKYKY residues were converted into DOPA-Lys-DOPA-Lys-DOPA (XKXKX), the increase of adsorption capacities and the hydrophilicity on the surface of PLGA films both affected hUCMSCs adhesion and paracrine activity. This gene level of NGF was further confirmed by western blot assessing protein level of the secreted medium from hUCMSCs cultured on different PLGA films. In this case, the highest expression was noticed for DOPA-IGF-1@PLGA samples, a pattern basically the same as that observed in gene levels, reflecting the importance of epigenetic steps of the stimulated genes.

Functional Effects of Neurotrophic Factors Secreted From hUCMSCs

To clarify the biological functions of the secreted neurotrophic factors of MSCs, particularly NGF, a model experiment was carried out by culturing PC12 cells using different CMs which were obtained from cultured hUCMSCs for 14 days. In this model, hUCMSCs were physically separated from PC12 cells, leaving interaction with PC12 cells via neurotrophic factors only. The morphology of PC12 cells was observed during incubation for 1, 3, and 6 days as shown in Figure 9. After culturing for 1 day, the neuritogenesis behavior was not observed; few neurites were generated with CM from DOPA-IGF-1@PLGA group. When cultured for 3 days, no obvious neurite outgrowth was observed in PC12 cells with MM and CM of PLGA group. Some neurites were somewhat generated with CM of cIGF-1@PLGA films, and a much higher degree of neurite outgrowth was noticed in the hUCMSCs with CM of Y-IGF-1@PLGA and DOPA-IGF-1@PLGA. With culture for 6 days, the neuritogenesis behavior was more evident for both CMs of Y-IGF-1@PLGA and DOPA-IGF-1@PLGA samples, especially for the later one. Neurite outgrowth patterns were analyzed systematically via number of cells with neurite outgrowth and average length of neurite outgrowth

(Fig. 10). With culture for 3 and 6 days, the cell number with neurite outgrowth for CM of DOPA-IGF-1@PLGA sample was slightly more than that of the other groups (Fig. 10a). The average neurite length of PC12 cells for CM of DOPA-IGF-1@PLGA sample was significant longer than all the other groups after 6 days of culture (Fig. 10b). The quantitative results of PC 12 cells cultured in the CMs obtained from hUCMSCs grown on the different modified substrates indicated that the neurotrophic factors secreted by the hUCMSCs could promote cell differentiation with neurite outgrowth. To further investigate the neural differentiation of PC12 cells, synaptophysin 1 (SYN1) and microtubule-associated protein 2 (MAP2) gene expression analyses were carried out for 3 and 6 days (Fig. 11). SYN1 is a major synaptic vesicle membrane protein that is expressed in neurons.[48] MAP2 regulates tubulin polymerization and is cardinal in microtubule assembly.[49] The qRT-PCR analysis showed that there was no significant difference in SYN1 expression at 3 days. However, at 6 days, the SYN1 expression in PC12 cells cultured with CMs of cIGF-1@PLGA and Y-IGF-1@PLGA were significantly increased compared to control and PLGA groups ($p < 0.05$). It was also evident that the expression of SYN1 for DOPA-IGF-1@PLGA was the highest than the other groups ($p < 0.05$). As MAP2 expression for the CM of Y-IGF-1@PLGA sample was higher compared to control, PLGA, and cIGF-1@PLGA samples. Among all the samples, MAP2 expression of DOPA-IGF-1@PLGA group was the highest one at 3 days. At 6 days, this trend was more obviously observed ($p < 0.05$). This indicated that CM of DOPA-IGF-1@PLGA is more effective in eliciting neural differentiation. As neuritogenesis of PC12 cells is known to be very responsive to neurotrophic factors, particularly NGF.[50] It was reported that with the presence of NGF, PC12 cells cease division and differentiate into sympathetic-like neurons, producing long neuritis.[51] As hUCMSCs were cultured on different PLGA films, the amount of secreted growth factors was in accordance with the amount of IGF-1 on unit area of PLGA. The results of qRT-PCR and western blot analysis aforementioned indicated that the highest expression of neurotrophic factors could be achieved with efficient immobilization of DOPA-IGF-1 on the PLGA implants and it would be beneficial for neural repair and regeneration processes.

Conclusion

In this study, we used a novel fusion protein DOPA-IGF-1 with an exquisite binding ability to promote cellularization and paracrine activity. DOPA-IGF-1 was dependable and immobilized efficiently onto PLGA films. The cultured hUCMSCs within this sample not only exhibited exquisite ability to promote cell proliferation and adhesion but also supported neurotrophic factor secretion, showing significant bioactivity on neurite outgrowth of PC12 cells. Both neurotrophic factor secretion capacity and bioactivity of the hUCMSCs on the DOPA-IGF-1@PLGA were found to be significantly higher than that of their cIGF-1 and Y-IGF-1 counterpart. Overall, it can be concluded that DOPA-IGF-1—mobilized PLGA substrates to support neurotrophic factor secretion of hUCMSCs in this study may provide an attractive technology for neural regeneration applications.

Acknowledgments

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References

1. Sakiyama-Elbert SE, Panitch A, Hubbell JA. Development of growth factor fusion proteins for cell-triggered drug delivery. *FASEB J*. 2001;15(7):1300-1302.
2. Assuncao-Silva RC, Gomes ED, Sousa N, Silva NA, Salgado AJ. Hydrogels and cell based therapies in spinal cord injury regeneration. *Stem Cells Int*. 2015;2015:948040.
3. Schmidt CE, Leach JB. Neural tissue engineering: strategies for repair and regeneration. *Annu Rev Biomed Eng*. 2003;5:293-347.
4. Cui Y, Liu Y, Cui Y, Jing X, Zhang P, Chen X. The nanocomposite scaffold of poly(lactide-co-glycolide) and hydroxyapatite surface-grafted with L-lactic acid oligomer for bone repair. *Acta Biomater*. 2009;5(7):2680-2692.
5. Teng YD, Lavik EB, Qu XL, et al. Functional recovery following traumatic spinal cord injury mediated by a unique polymer scaffold seeded with neural stem cells (vol 99, pg 3024, 2002). *Proc Natl Acad Sci U S A*. 2002;99(14):9606.
6. Lee JY, Bashur CA, Goldstein AS, Schmidt CE. Polypyrrole-coated electrospun PLGA nanofibers for neural tissue applications. *Biomaterials*. 2009;30(26):4325-4335.
7. Chang C-J, Hsu S-H, Yen H-J, Chang H, Hsu S-K. Effects of unidirectional permeability in asymmetric poly(DL-lactic acid-co-glycolic acid) conduits on peripheral nerve regeneration: an in vitro and in vivo study. *J Biomed Mater Res B Appl Biomater*. 2007;83B(1):206-215.
8. Huang Y-C, Huang C-C, Huang Y-Y, Chen K-S. Surface modification and characterization of chitosan or PLGA membrane with laminin by chemical and oxygen plasma treatment for neural regeneration. *J Biomed Mater Res A*. 2007;82-A(4):842-851.
9. Lavik E, Teng YD, Snyder E, Langer R. Seeding neural stem cells on scaffolds of PGA, PLA, and their copolymers. *Methods Mol Biol*. 2002;198:89-97.
10. Grasel TG, Pierce JA, Cooper SL. Effects of alkyl grafting on surface-properties and blood compatibility of polyurethane block copolymers. *J Biomed Mater Res*. 1987;21(7):815-842.
11. Policastro GM, Lin F, Callahan LAS, et al. OGP functionalized phenylalanine-based poly(ester urea) for enhancing osteoinductive potential of human mesenchymal stem cells. *Biomacromolecules*. 2015;16(4):1358-1371.
12. Tada S, Kitajima T, Ito Y. Design and synthesis of binding growth factors. *Int J Mol Sci*. 2012;13(5):6053-6072.
13. Joddar B, Ito Y. Biological modifications of materials surfaces with proteins for regenerative medicine. *J Mater Chem*. 2011;21(36):13737-13755.
14. Sun W, Lin H, Chen B, Zhao W, Zhao Y, Dai J. Promotion of peripheral nerve growth by collagen scaffolds loaded with collagen-targeting human nerve growth factor-beta. *J Biomed Mater Res A*. 2007;83A(4):1054-1061.
15. Kitajima T, Sakuragi M, Hasuda H, Ozu T, Ito Y. A chimeric epidermal growth factor with fibrin affinity promotes repair of injured keratinocyte sheets. *Acta Biomater*. 2009;5(7):2623-2632.
16. Li XY, Wang Y, Guo M, et al. Degradable three dimensional-printed polylactic acid scaffold with long-term antibacterial activity. *ACS Sustainable Chem Eng*. 2018;6(2):2047-2054.
17. Yang K, Park E, Lee JS, et al. Biodegradable nanotopography combined with neurotrophic signals enhances contact guidance and neuronal differentiation of human neural stem cells. *Macromol Biosci*. 2015;15(10):1348-1356.
18. Li G. A nanoparticle-based model system for investigating the molecular mechanism of surface charges on migration and proliferation of schwann cells in peripheral nerve regeneration. *Nanomed Nanotechnol Biol Med*. 2016;12(2):475.
19. Zhang C, Miyatake H, Wang Y, et al. A bioorthogonal approach for the preparation of a titanium-binding insulin-like growth-factor-1 derivative by using tyrosinase. *Angew Chem Int Ed Engl*. 2016;55(38):11447-11451.
20. O'Kusky J, Ye P. Neurodevelopmental effects of insulin-like growth factor signaling. *Front Neuroendocrinol*. 2012;33(3):230-251.
21. Arsenijevic Y, Weiss S, Schneider B, Aebischer P. Insulin-like growth factor-1 is necessary for neural stem cell proliferation and demonstrates distinct actions of epidermal growth factor and fibroblast growth factor-2. *J Neurosci*. 2001;21(18):7194-7202.
22. Youssef A, Aboalola D, Han VKM. The roles of insulin-like growth factors in mesenchymal stem cell niche. *Stem Cells Int*. 2017;2017:9453108.
23. Long XX, Olszewski M, Huang W, Kletzel M. Neural cell differentiation in vitro from adult human bone marrow mesenchymal stem cells. *Stem Cells Dev*. 2005;14(1):65-69.
24. Kim H-J, Lee J-H, Kim S-H. Therapeutic effects of human mesenchymal stem cells on traumatic brain injury in rats: secretion of neurotrophic factors and inhibition of apoptosis. *J Neurotrauma*. 2010;27(1):131-138.
25. Bongso A, Fong C-Y. The therapeutic potential, challenges and future clinical directions of stem cells from the wharton's jelly of the human umbilical cord. *Stem Cell Rev Rep*. 2013;9(2):226-240.
26. Lee JH, Lee J-Y, Yang SH, Lee E-J, Kim H-W. Carbon nanotube-collagen three-dimensional culture of mesenchymal stem cells promotes expression of neural phenotypes and secretion of neurotrophic factors. *Acta Biomater*. 2014;10(10):4425-4436.
27. Hong Z, Zhang P, Liu A, Chen L, Chen X, Jing X. Composites of poly(lactide-co-glycolide) and the surface modified carbonated hydroxyapatite nanoparticles. *J Biomed Mater Res A*. 2007;81-A(3):515-522.
28. Wang Y, Fu C, Wu Z, et al. A chitin film containing basic fibroblast growth factor with a chitin-binding domain as wound dressings. *Carbohydr Polym*. 2017;174:723-730.

29. Mitchell KE, Weiss ML, Mitchell BM, et al. Matrix cells from Wharton's jelly form neurons and glia. *Stem Cells*. 2003;21(1):50-60.
30. Dikmen M. Comparison of the effects of curcumin and RG108 on NGF-induced PC-12 adh cell differentiation and neurite outgrowth. *J Med Food*. 2017;20(4): 376-384.
31. Keefe KM, Sheikh IS, Smith GM. Targeting neurotrophins to specific populations of neurons: NGF, BDNF, and NT-3 and their relevance for treatment of spinal cord injury. *Int J Mol Sci*. 2017;18(3):548-565.
32. Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. *Physiol Rev*. 2003;83(3):835-870.
33. Liu Y, Ai K, Lu L. Polydopamine and its derivative materials: synthesis and promising applications in energy, environmental, and biomedical fields. *Chem Rev*. 2014;114(9):5057-5115.
34. Ko E, Yang K, Shin J, Cho S-W. Polydopamine-assisted osteoinductive peptide immobilization of polymer scaffolds for enhanced bone regeneration by human adipose-derived stem cells. *Biomacromolecules*. 2013;14(9):3202-3213.
35. Mazia D, Schatten G, Sale W. Adhesion of cells to surfaces coated with polylysine - applications to electron-microscopy. *J Cell Biol*. 1975;66(1): 198-200.
36. Wang Z, Chen L, Wang Y, Chen X, Zhang P. Improved cell adhesion and osteogenesis of op-HA/PLGA composite by poly(dopamine)-assisted immobilization of collagen mimetic peptide and osteogenic growth peptide. *ACS Appl Mater Inter*. 2016;8(40):26559-26569.
37. Gao T, Zhang N, Wang Z, et al. Biodegradable microcarriers of poly(lactide-co-glycolide) and nano-hydroxyapatite decorated with IGF-1 via polydopamine coating for enhancing cell proliferation and osteogenic differentiation. *Macromol Biosci*. 2015;15(8):1070-1080.
38. Chien CY, Tsai WB. Poly(dopamine)-assisted immobilization of Arg-Gly-Asp peptides, hydroxyapatite, and bone morphogenic protein-2 on titanium to improve the osteogenesis of bone marrow stem cells. *Acs Appl Mater Inter*. 2013;5(15):6975-6983.
39. Van Lonkhuyzen DR, Hollier BG, Shooter GK, Leavesley DI, Upton Z. Chimeric vitronectin : insulin-like growth factor proteins enhance cell growth and migration through co-activation of receptors. *Growth Factors*. 2007;25(5):295-308.
40. Kim EY, Tripathy N, Cho SA, Lee D, Khang G. Collagen type I-PLGA film as an efficient substratum for corneal endothelial cells regeneration. *J Tissue Eng Regen Med*. 2017;11(9):2471-2478.
41. Fu C, Yang X, Tan S, Song L. Enhancing cell proliferation and osteogenic differentiation of MC3T3-E1 pre-osteoblasts by BMP-2 delivery in graphene oxide-incorporated PLGA/HA biodegradable microcarriers. *Sci Rep*. 2017;7:12549.
42. Reeves A, Keirstead HS. Stem cell based strategies for spinal cord injury repair. *Adv Exp Med Biol*. 2012;760:16-24.
43. Doorn J, Roberts SJ, Hilderink J, et al. Insulin-like growth factor-i enhances proliferation and differentiation of human mesenchymal stromal cells *in vitro*. *Tissue Eng Part A*. 2013;19(15-16):1817-1828.
44. Hawryluk GWJ, Mothe A, Wang J, Wang S, Tator C, Fehlings MG. An *in vivo* characterization of trophic factor production following neural precursor cell or bone marrow stromal cell transplantation for spinal cord injury. *Stem Cells Dev*. 2012;21(12):2222-2238.
45. Uz M, Buyukoz M, Sharma AD, Sakaguchi DS, Altinkaya SA, Mallapragada SK. Gelatin-based 3D conduits for transdifferentiation of mesenchymal stem cells into Schwann cell-like phenotypes. *Acta Biomater*. 2017;53:293-306.
46. Tohill M, Terenghi G. Stem-cell plasticity and therapy for injuries of the peripheral nervous system. *Biotechnol Appl Biochem*. 2004;40:17-24.
47. Forbes K, Westwood M. The IGF axis and placental function. *Horm Res*. 2008;69(3):129-137.
48. Bergmann M, Schuster T, Grabs D, et al. Synaptophysin and synaptoporin expression in the developing rat olfactory system. *Brain Res Dev Brain Res*. 1993;74(2):235-244.
49. Brugg B, Matus A. PC12 cells express juvenile microtubule-associated proteins during nerve growth factor-induced neurite outgrowth. *J Cell Biol*. 1988;107(2):643-650.
50. Jacovina AT, Zhong FM, Khazanova E, Lev E, Deora AB, Hajjar KA. Neuritogenesis and the nerve growth factor-induced differentiation of PC-12 cells requires annexin II-mediated plasmin generation. *J Biol Chem*. 2001;276(52):49350-49358.
51. Pollock JD, Krempin M, Rudy B. Differential-effects of NGF, FGF, EGF, CAMP, and dexamethasone on neurite outgrowth and sodium-channel expression in PC12 cells. *J Neurosci*. 1990;10(8):2626-2637.