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Purmorphamine as a Shh Signaling Activator Small Molecule Promotes Motor Neuron Differentiation of Mesenchymal Stem Cells Cultured on Nanofibrous PCL Scaffold

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Abstract There is variety of stem cell sources but problems in ethical issues, contamination, and normal karyotype cause many limitations in obtaining and using these cells. The cells in Wharton's jelly region of umbilical cord are abundant and available stem cells with low immunological incompatibility, which could be considered for cell replacement therapy. Small molecules have been presented as less expensive biologically active compounds that can regulate different developmental process. Purmorphamine (PMA) is a small molecule that, according to some studies, possesses certain differentiation effects. In this study, we investigated the effect of the PMA on Wharton's jelly mesenchymal stem cell (WJ-MSC) differentiation into motor neuronal lineages instead of sonic hedgehog (Shh) on PCL scaffold. After exposing to induction media for 15 days, the cells were characterized for expression of motor neuron markers including PAX6, NF-H, Islet1, HB9, and choline acetyl transferase (ChAT) by quantitative reverse transcription (PCR) and immunocytochemistry. Our results demonstrated that induced WJ-MSCs with PMA could significantly express motor neuron markers in RNA and protein levels 15 days post induction. These results suggested that WJ-MSCs can differentiate to motor neuron-like cells with PMA on PCL scaffold and might provide a potential source in cell therapy for nervous system.

Keywords Motor neuron differentiation · Wharton's jelly mesenchymal stem cells · Purmorphamine · PCL scaffold

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Introduction

Neuronal cell loss is common feature for large numbers of neurodegenerative diseases such as Parkinson's disease, spinal cord injury, stroke, and Huntington's disease (HD) that are associated with loss of function and disabilities [1–3]. There are many limitations in injured regions in the CNS, and this problem greatly limits effective therapeutic possibilities [4]. Cell replacement therapy for injured regions has provided powerful new therapeutic strategies for human neurological diseases [1, 5]. There are many sources of stem cells that are used for neural differentiation [6, 7]. Embryonic stem cells (ESCs) and adult stem cells have been differentiated into neural cells that are used for repairing of neurodegenerative diseases [8–12]. For clinical application, it is important that these cells have the relative ease of isolating

and expanding process [13]. Conversely, mesenchymal stem cells derived from Wharton's jelly mesenchymal stem cells (WJ-MSCs) within the umbilical cord are easy to be obtained and do not give rise to any ethical issues, not tumorigenic, and preserve their normal karyotype after several passages [14–18]. Previously, the differentiation potential of this source of cells into many types of cells such as osteoblast, adipocyte, and neural cells has been studied [16, 19, 20]. Thus, human umbilical cord mesenchymal stem cells may serve as an alternative source of multipotent stem cells for replacement therapy [16]. It is previously shown that WJ-MSCs can be differentiated to motor neurons by applying retinoic acid (RA) and sonic hedgehog (Shh) [16, 18]. In the present study, we also discovered that motor neuron differentiation from WJ-MSCs can be achieved by using a small molecule, purmorphamine, instead of Shh. Recently, the effect of a small molecule termed 2,6,9-trisubstituted purine or purmorphamine has investigated on osteogenesis and neurogenesis by activation of the hedgehog signaling pathway [21–24]. Upregulation of Gli-1 transcription factor is important for neural differentiation that can be occurred by activation of Shh signaling pathway by purmorphamine [25]. Identification of small molecules that selectively induce neural differentiation from MSCs would provide useful chemical tools to study the molecular mechanisms of neural differentiation and ultimately might lead to useful therapeutic agents for the treatment of neurodegenerative diseases [26].

Tissue engineering is a new potential approach to enhance cell survival and differentiation using materials. A variety of natural and synthetic polymers for nerve tissue engineering have been investigated by variety of studies such as polycaprolactone (PCL), poly(L-lactide-co-glycolide) (PLGA), collagen, and gelatin [16, 27]. PCL has good mechanical properties, biodegradability, and biocompatibility, which has been shown to be suitable for constructing scaffold for differentiation of stem cells to different types of cells such as neural cells [16, 27]. Among different methods for scaffold fabrication, electrospinning has drawn attention because it is an easy, cost-effective technique and provides a proper matrix with high surface area to volume ratio and fiber diameters in the range of nanometer with sufficient pores to encourage mesenchymal stem cell (MSC) adhesion, proliferation, and differentiation [28].

In the present study, we investigated the directed differentiation of human WJ-MSCs cultured on PCL nanofibrous scaffold into motor neuron-like cells in the presence of purmorphamine as an agonist of Shh signaling. PCL nanofibrous scaffold was fabricated by electrospinning technique, and then differentiation of WJ-MSCs into motor neuron cells on PCL scaffold was characterized by investigating their morphology and specific gene expression.

Material and Methods

Mesenchymal Stem Cell Isolation from Wharton's Jelly

Human WJ-MSCs were obtained by our previous study protocol [17]. Briefly, the sample of human umbilical cords (hUCs) was collected after filling consent forms by the newborns' parent. Cord was rinsed with PBS to remove blood in excess and was processed within 2–4 h of the births. After that, hUC was cut into 3–5-cm pieces, and cord vessels were removed to avoid endothelial cell contamination. Wharton's jelly parts were digested with collagenase I (1 mg/ml; Sigma-Aldrich, USA) for 3 h at 37 °C and further digested with dispase enzyme at 37 °C for 1 h and then centrifuged at 1500 rpm for 5 min at 4 °C. After discarding the liquid, isolated cells were cultured in Dulbecco's modified Eagle medium/F12 (DMEM/F12; Invitrogen, USA), 10 % fetal bovine serum (Invitrogen USA), 100 U/ml penicillin/streptomycin (Sigma, USA) and plated into a 25-cm^2 flask and maintained at 37 °C in a 5 % CO incubator. Medium was renewed every 3 days, and adherent cells were serially passaged at 80–90 % confluence at 1:3 ratio. WJ-MSCs were characterized using flow cytometry for cell surface markers including CD90, CD105, CD73, CD45, and CD34 [17].

Fabrication of Electrospun PCL

To obtain nanofibrous scaffolds, PCL polymer (10 % *w*/*v*; Mw 80,000 g/mol, Sigma-Aldrich, USA) was dissolved in mixture of dichloromethane (DCM), *N*,*N*-dimethylformamide (DMF) at the ratio of 1:3 and stirred for 24 h at room temperature. The polymer solution was placed into a 5-ml plastic syringe at room temperature. Applied voltage was 15 kV and the feeding rates were kept constant at 1.3 ml/h. As grounded collector, a piece of aluminum foil was placed toward the tip at the distance of 12 cm.

Cell Seeding on PCL Scaffolds

WJ-MSCs were cultured in DMEM/F12 (Gibco, USA) supplemented with 10 % FBS. When the confluence of cells reached 85–90 % at passage 3, the cells were used for seeding on scaffolds. The scaffolds were cut to the size of a well from a 24-well plate (16 mm) using a punch. Scaffolds were sterilized by exposing to UV radiation for 1 h and incubated in DMEM/F12 containing 10 % FBS for 2 h before cell seeding. The cells were dropped onto the top of the scaffolds with a final seeding density of 5×10^4 cells/cm^2 of scaffold in 24-well plates and incubated for 2 h to allow cells to attach onto the surface of the scaffold. New medium was then added for further incubation.

Scanning Electron Microscopy

The average of fiber diameter and diameter distribution of the fibers were measured by analyzing of SEM micrographs by using the Image J software from 50 fibers per condition. To SEM of WJ-MSCs cultured on nanofibrous scaffolds for 5 days, cell-containing scaffolds were fixed with 2.5 % glutaraldehyde for 1 h and dehydrated in series of sequentially increasing concentration of ethanol solutions (30, 50, 70, 80, 90, and 100 %) for 10 min per each concentration. Critical point-dried samples were sputter-coated with gold and examined using a scanning electron microscope (model Philips XL-30, Netherland), operated at 15 kV.

Cell Viability and Proliferation Assay

The 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to measure metabolic activity of the WJ-MSCs cultured on the electrospun PCL nanofibrous scaffolds. Cells were seeded at a density of 5×10^4 cells/scaffold in 24-well plates and incubated at 37 °C under 5 % CO_2 for 1, 3, 5, and 7 days. For this assay, 400 μl of 5 mg/ml MTT solution was added to each well and incubated at 37 °C for 4 h. The medium was removed, the formazan crystals were dissolved in DMSO, and absorbance at 570 nm was measured using an ELIZA reader (Asys Hitch, Ec Austria).

Induction of WJ-MSCs into Motor Neuron-Like Cells with purmorphamine

For motor neuron-like cell induction, WJ-MSCs were seeded at 5000 cells/cm^2 on PCL scaffolds as 3D culture and 24-well tissue culture polystyrene (TCP). The cells were incubated with DMEM/F12 medium supplemented with 10 % FBS, 100 U/ml penicillin, and 1 mg/ml streptomycin for 24 h. Differentiation of cells was induced by exposing the cells to preinduction medium composed of DMEM/F12 (1:1), 20 % FBS, 2 % B27, 10 ng/ml fibroblast growth factor 2 (FGF2), 250 μM isobutylmethylxanthin, and 100 μM 2-metcaptoethanol and incubated for 24 h at 37 °C and 5 % CO_2. The treated cells were then cultured in induction media containing DMEM/F12 (1:1), 0.2 % B27, 1 μM of purmorphamine (PMA), and 0.01 ng/ml retinoic acid (RA) for 1 week. Then, the induced media was replaced with a medium composed of DMEM/F12 (1:1), 0.2 % B27, and 200 ng/ml brain-derived neurotrophic factor (BDNF) for another 1 week. As a control, a group of WJSCs was cultured on the 3D cultures or 2D cultures in the absence of differentiation factors for 15 days. The medium was changed every 3 days.

Immunofluorescence Analysis

After induction to motor neuron cells, cells were fixed with 4 % paraformaldehyde (PFA; Sigma-Aldrich) and permeabilized with 0.1 % TX-100 in TBS. The cells were blocked for 30 min at room temperature with 5 % BSA; incubated with primary antibodies against NF-H(SMI-32) (mouse monoclonal antihuman; Abcam, USA, 1:200), beta-tubulin III (mouse monoclonal antihuman; Abcam, 1:200), choline acetyltransferase (Chat) (mouse monoclonal antihuman; Abcam,1:200), and Islet-1 (mouse monoclonal antihuman; Abcam, 1:200); and diluted in 5 % BSA in PBS overnight. Secondary antibodies included Alexa fluor 488 donkey anti-mouse (1:500; Gibco, A-11058) or Alexa Fluor 594 donkey anti-rabbit (1:700; Gibco, A-21207), and the nuclei were counterstained with DAPI (Sigma-Aldrich, D8417). For negative controls, only the secondary antibodies were used. To quantify the number of positive cells for each antibody, at least ten microscopic fields per well were counted randomly and reported in relative to whole DAPI-stained nuclei as percentage.

Molecular Analysis Using Real-Time-PCR

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was used for the mRNA expression patterns of neuronal markers in treatment groups. Total RNA was extracted by using RNeasy Plus Mini kit (Qiagen, USA, 74134), and complementary DNA (cDNA) synthesis from 1 μg of RNA was performed by Revert Aid First Strand cDNA Synthesis kit (Takara, USA, K1632). qRT-PCR reactions were carried out in the 48-well optical reaction plates on StepOneTM Real-Time PCR machine. In each PCR reaction, 30 ng synthesized cDNA was used to PCR by mixing with 10 μl of Power SYBER Green master mix (2×, Applied Biosystems), 0.5 μM of each primer (Table 1) in a total volume of 20 μl at the annealing temperature. The comparative Ct method, 2−DDCt, was used for relative gene expression analysis.

Statistical Analysis

The data were presented as means ± standard deviation of the means ($n = 3$). Statistical analysis was carried out using one-way ANOVA, and difference between groups was considered statistically significant if $P > 0.05$.

Results

Isolation and Identification of Human MSCs Derived from Wharton's Jelly

Flow cytometry analysis which was published in our previous report (17) showed that CD90+ (93.6), CD105+ (90.7), and

Table 1 Primers used for real-time RT-PCR

Gene	Primer sequence(5′–3′)	Annealing (°C)
Nestin	F AAAGTTCCAGCTGGCTGTGG	55
	R TCCAGCTTGGGGTCCTGAAA	
Pax-6	F CGGTTTCCTCCTTCACAT	50
	R ATCATAACTCCGCCCATT	
Islet 1	F ATATCAGGTTGTACGGGTCAAAT	56
	R CACGCATCACGAAGTCGTTC	
Chat	F GCAGGAGAAGACAGCCAACT	55
	R AAACCTCAGCTGGTCAT	
NF-H	F CAGAGCTGGAGGCACTGAAA	55
	R CTGCTGAATGGCTTCCTGGT	
Hb9	F AGCACCAGTTCAAGCTCAACA	55
	R ACCAAATCTTCACCTGGGTCTC	
GAPDH	F TCGCCAGCCGAGCCA	55
	R CCTTGACGGTGCCATGGAAT	

CD73+ (89.8) were highly expressed in hWJ-MSCs and cells were negative for CD34 and CD4 (hematopoietic lineage markers).

Fabrication of PCL Electrospun Nanofibrous Scaffolds

PCL nanofibers fabricated by electrospining technique were used in this study. Electrospinning of PCL nanofibers was homogeneous without any bead and branching. Scanning electron microscopy micrographs showed that the average diameter of fibers was 100 nm as shown in Fig. 1a, b. The morphology and the interaction between cells and PCL electrospun scaffold were scanned at 3 days after cell seeding. Images demonstrated that cells attached, grew, and spread on the PCL nanofibrous scaffolds (Fig. 1a, b).

Assessment of Cell Adhesion and Viability

MTT assay was performed to investigate the viability of cells cultured on PCL scaffolds as 3D group and TCP as 2D culture, at 1, 3, 5, and 7 days. As shown in Fig. 1c, until day 3, the WJ-MSCs cultured on TCP and PCL scaffold showed higher viability than cells cultured on the PCL scaffold but was not statistically significant. However, in days 5 and 7, the viability of WJ-MSCs cultured on PCL scaffold significantly enhanced relative to cells cultured on 2D control group (Fig. 2). The results obtained from MTT assay showed that PCL nanofibrous scaffolds were suitable substrates than TCPs in relation to cell attachment and proliferation.

Evaluation of WJ-MSC Differentiation into Motor Neuron-Like Cell by PMA

To confirm motor neuron-like cell differentiation, expression of neural markers including NF-H, Chat, and Islet-1 was investigated by immunofluorescence staining at day 15. Figure 3 shows that the random field counting of immunofluorescence images shows the percentage. Our results showed a higher expression of NF-H, choline acetyltransferase (Chat), and Islet-1 in two groups. Neuronal cell marker expression in TCP group was compared with PCL group. The expression of NF-H (50 %), Chat (78 %), and Islet-1 (82 %) in the PCL group was higher than the expression of NF-H (42 %), Chat (61 %), and Islet-1 (76 %) in the TCP group, but the expression of Chat in the PCL group in comparison with the TCP group was statistically significant (Fig. 3). The mRNA expression of neural specific genes was observed during WJ-MSC differentiation by qRT-PCR at day 15 of induction for TCP and PCL scaffold groups. The expression of nestin, Pax6, Islet-1, Chat, NF-H, and HB9 mRNAs was compared in TCP and PCL groups vs undifferentiated WJ-MSCs as a control group. According to our results, while the expression of Islet-1, Chat, NF-H, and HB9 increased significantly during differentiation, the expression of nestin and Pax6 was down-regulated following induction. The comparison results between TCP and PCL groups showed that in the PCL group, the expression of Islet-1($P > 0.01$), Chat ($P > 0.05$), NF-H ($P > 0.05$), and HB9 ($P > 0.01$) was higher than in the PCL group and was statistically significant as shown in Fig. 4. These results confirmed that treatment with PMA could induce differentiation of WJ-MSCs into motor neuron-like cells and PCL nanofibrous provides a suitable condition to cell differentiation.

Discussion

The main goal of this study was to indicate the capability of cultured WJ-MSCs on PCL nanofibrous scaffold to differentiate into motor neuron-like cells in the presence of PMA as a small molecule that mimics Shh protein. In this study, we found that stimulation of hedgehog signaling pathway by means of PMA leads to promotion of WJ-MSC differentiation to motor neuron-like cells. Expression of specific markers such as beta-tubulin III, Chat, Islet-1, NF-H, HB9, Pax6, and nestin in protein and mRNA levels was analyzed using real-time PCR and immunocytochemistry, respectively.

The results of immunocytochemistry and real-time PCR represented the higher amount of differentiation to motor neuron-like cells in cultured cells on PCL scaffold relative to those on a TPC surface (2D control group).

It has been demonstrated that biomaterials are designed to create a niche that provides the appropriate microenvironment to enhance cell survival [29–32]. In line with previous studies, our MTT assay results showed that the viability of WJ-MSCs cultured on PCL scaffold highly increased relative to cells cultured on TPC surface.

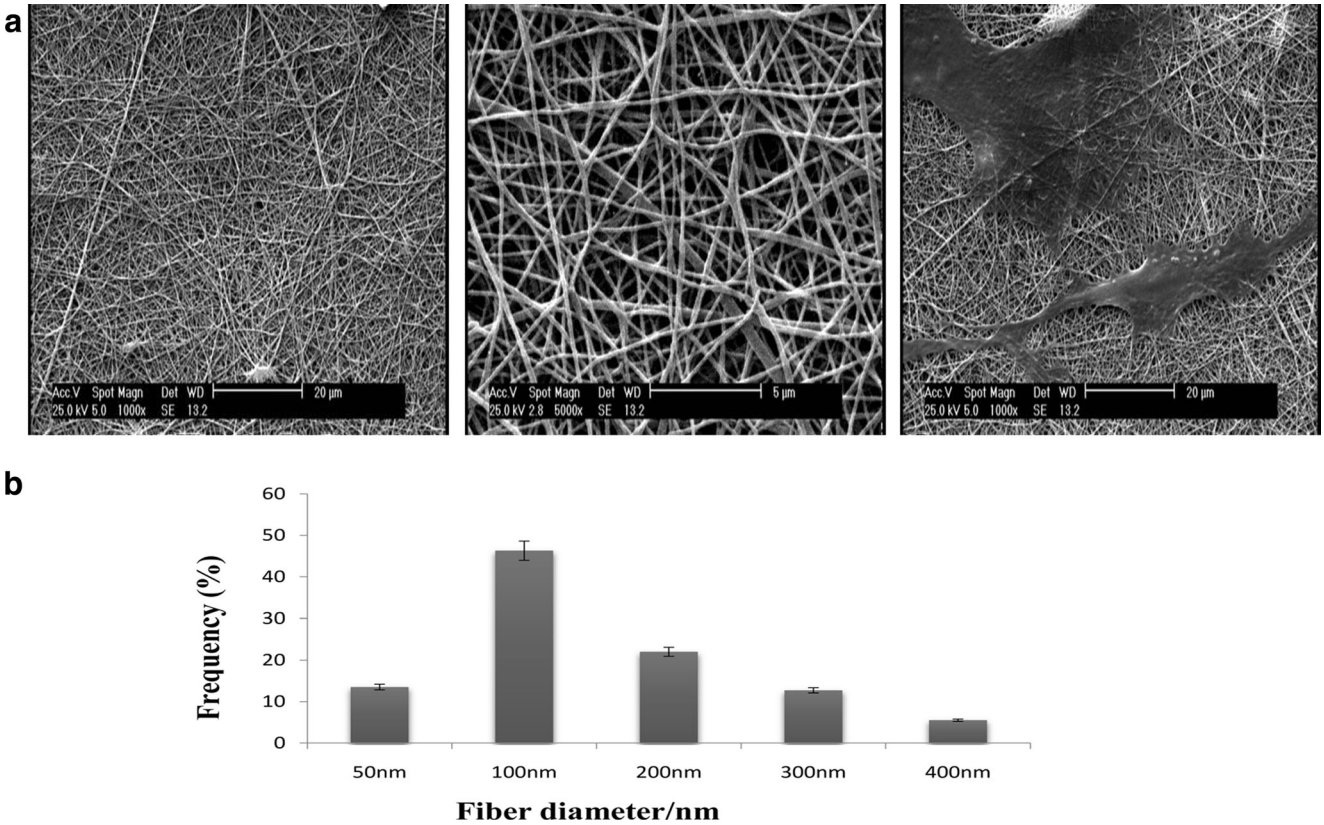

Fig. 1 Scaffold characterization analysis. **a** Scanning electron micrographs showing PCL scaffolds with and without cells. The fibers of PCL scaffold were randomly entangled to form a flexible and porous 3D matrix (*scale bar* 20 and 5 μm). Plated cells on PCL scaffold that grew on PCL scaffold 5 days after seeding (*scale bar* 20 mm). **b** Histogram shows diameters of the PCL nanofibers. The average of fiber diameter and diameter distribution of the fibers was measured by analyzing SEM micrographs by measuring 50 fibers per condition using the ImageJ software

Importantly, for those kinds of neurological disorders [33–35] that motor neurons are affected [36], the generated neurons may need to contain a large population of motor neurons that can enhance therapeutic efficacy [37]. Thus, signaling pathways that induce differentiation of motor neurons, such as Shh, can potentially improve the efficiency of cell transplants, as overall, fewer cells need to be injected because of a higher motor neuronal yield [38, 39].

Shh is known to promote MSC differentiation into motor neuron-like cells [10, 17]. The previous studies have demonstrated that Shh signaling pathway plays a critical role in development and generation of motor neuron in embryo [40].

Enhancing of neuronal differentiation can be obtained by using chemical factors that stimulate signaling pathways controlling the development of neurons [41]. Moreover, these factors present an opportunity to provide more control over

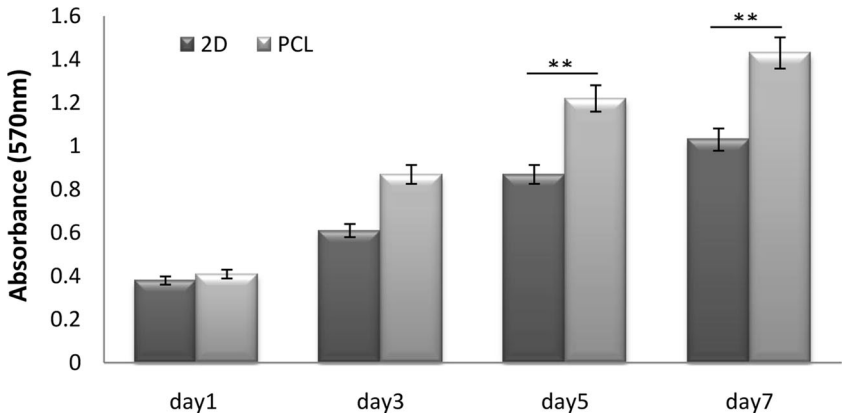

Fig. 2 MTT assay. Formosan absorbance expressed as a measure of cell viability from the cell cultured on TCP (2D) and PCL (3D) nanofibrous scaffolds for 7 days

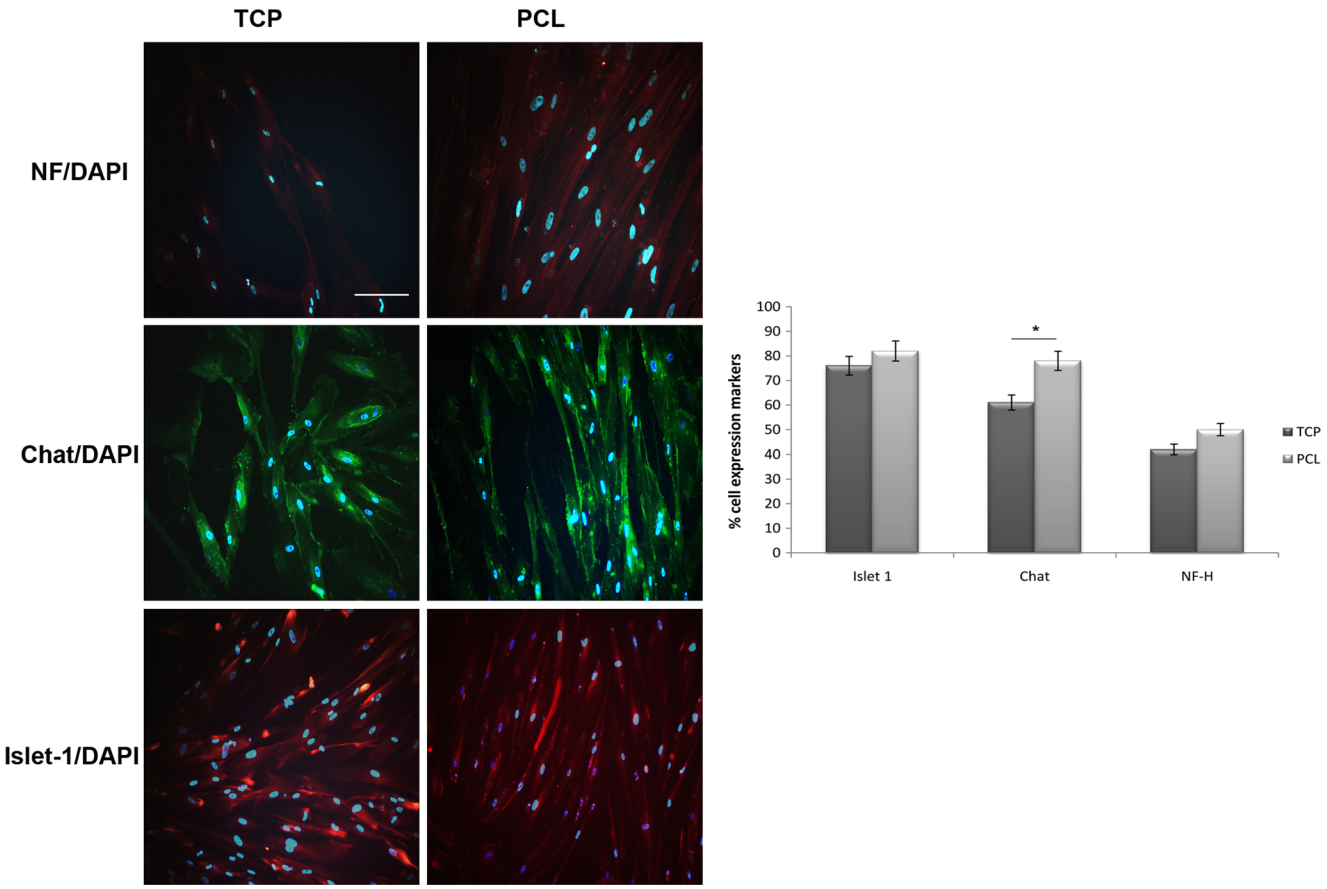

Fig. 3 Immunofluorescence staining of differentiated cells on TCP and PCL scaffolds after 15 days post induction for motor neuron markers including Chat, Islet-1, and NF-H. *Scale bar* 50 μm. Expression (ratio of positive cells, %) of motor neuron markers after 15 days induction. Data are expressed as mean ± SD; three wells (ten fields per well) in each group. $*P < 0.05$ vs TCP group and PCL group

the neuronal fate of transplanted cells [42]. PMA, a synthetic small molecule, may also improve neuronal differentiation [21, 43] through activation of the smoothened receptor, which directly acts on the same signaling pathway as Shh [44]. One great advantage of small synthetic molecules, such as PMA, compared with naturally occurring molecules, such as Shh, is their stability [45]. PMA acts on the SMO receptor, binding of PMA to this receptor lead to increase of Gli1 expression, which is downstream of the Hedgehog pathway [43, 46]. Moreover, it has been demonstrated that Gli1 also has a neuroprotective effect on dopaminergic neurons in experimental models of neurodegenerative diseases [47]. Additionally, there is also a growing body of evidence about the activation of pro-survival signaling pathways such as autophagy under control of Shh pathway [47, 48]. Autophagy pathway plays a critical role in maintenance of neural precursor cells [49]. Thus, activation of Shh pathway by means of PMA may lead to an increase of cellular viability during differentiation of WJ-MSCs into motor neuron-like cells.

Indeed, action of PMA on the Hedgehog pathway through SMO is necessary to induce neuronal differentiation [50, 51]. Furthermore, it has shown that PMA not only increased

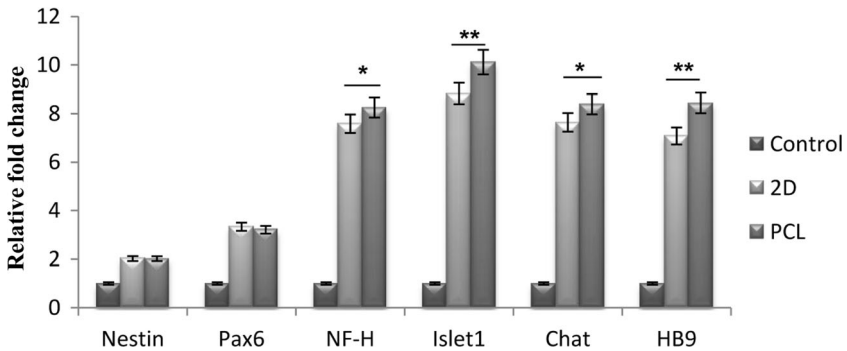

Fig. 4 Quantitative mRNA expression analysis of motor neuron-like cells derived from WJ-MSCs seeded on PCL scaffold after 15 days. The result of mRNA expression on TCP and PCL compared to undifferentiated WJ-MSCs. $*P > 0.05$, $**P > 0.01$ vs control ($n = 3$ biological samples, mean ± SEM)

neuronal differentiation but also increased the speed of differentiation [37]. In accordance with previous achievements, our results showed that PMA dramatically promotes differentiation of WJ-MSCs into motor neuron-like cells.

Conclusion

In conclusion, the results of this study appear that PMA can be used for MSC differentiation towards a motor neuron phenotype. Expression of motor neuron markers such as Chat, Islet-1, and NF-H in RNA and protein levels by real-time PCR and immunocytochemistry showed that PMA, a synthetic small molecule, promotes differentiation of WJ-MSCs into motor neuron-like cells cultured on PCL scaffold. Therefore, our achievements lead to a novel strategy for motor neuron derivation form WJ-MSCs in vitro and hence would provide an enabling tool for stem cell-based therapy of neurological disorders in the future.

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