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Neural progenitor cells derived from adult bone marrow mesenchymal stem cells promote neuronal regeneration

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ABSTRACT

Aim: It is well known that neural stem/progenitor cells (NS/PC) are an ideal cell type for the treatment of central nervous system (CNS) disease. However, ethical problems have severely hampered fetal NS/PC from being widely used as a source for stem cell therapy. Recently, it has been demonstrated that autologous bone marrow mesenchymal stem cells (BMSC) can transdifferentiate into neural progenitor cells (NPC). The biological function of BMSC derived NPC (MDNPC) in neuronal systems remains unknown. In the present study, we aimed to investigate whether MDNPC can promote *in vitro* neural regeneration, a process comprising mainly the generation of neurons and neurotransmitters.
Main methods: We co-cultured BMSC, MDNPC or fetal NS/PC with PC12 cells and studied their roles on proliferation, neurite formation and dopamine release from PC12 cells. Furthermore, we also explored the mechanisms by which MDNPC regulate dopamine secretion from PC12 derived neural cells using Western blot.
Key findings: We found that both MDNPC and NS/PC had similar morphologies and there were no significant differences between MDNPC and NS/PC in promoting PC12 cell proliferation, neurite outgrowth, and dopamine release. We also demonstrated that NS/PC induced dopamine secretion was associated with an upregulation of dopamine transporter (DAT) levels.
Significance: In summary, MDNPC were comparable to NS/PC in promoting neural regeneration, indicating that MDNPC are a promising candidate source of neural stem cells for the treatment of neurological diseases.

Introduction

The development of stem cell biology is crucial for regenerative medicine. Many different types of stem cells exhibit excellent therapeutic effects for improving the symptoms of various intractable neuronal diseases and the function of damaged neuronal tissue (Bae et al., 2007; Lu et al., 2001; Dezawa et al., 2005). This benefit is likely due to the ability of stem cells to secrete a range of molecules such as neurotrophic factors, anti-apoptotic factors and chemoattractants, which induce autologous stem cell migration and subsequent differentiation into neural cells (Joyce et al., 2010). However, the end-result or effects from stem cell therapy depend on the different origins and types of stem and progenitor cells employed (Bae et al., 2007). Many studies have demonstrated the use of neural stem/progenitor cells (NS/PC), which hold great potential for the treatment of various neurological diseases. Limited cell supply and ethical considerations limit the application of human NS/PC for clinical therapy. Therefore, it is important to continue exploring alternative sources of stem cells other than NS/PC with less ethical problems and greater therapeutic potential.

In recent years, numerous scientists have reported that bone marrow MSC (BMSC) can transdifferentiate into neuronal-like cells (Bae et al., 2007; Osaka et al., 2010; Jiang et al., 2003; Black and Woodbury, 2001). It has been well documented that a high percentage of nestin positive, BMSC derived neural progenitor cells (MDNPC) are intermediates during the early stage of transdifferentiation into neurons (Jiang et al., 2003; Black and Woodbury, 2001; Pittenger et al., 1999; Uccelli et al., 2008; Kopen et al., 1999). However, it is unknown whether the biological function and potential use of MDNPC will be clinically beneficial. To begin answering these questions, we co-cultured BMSC, MDNPC or fetal rat brain NS/PC with PC12 cells and compared their abilities to influence cell proliferation, neurite formation and dopamine release. The PC12 cell line is derived from a rat pheochromocytoma (Greene and Tischler, 1976).

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When treated with nerve growth factor (NGF), PC12 cells respond reversibly by acquiring a dopamine containing neuronal phenotype (Bae et al., 2007). This property makes PC12 cells a suitable model system for studying dopaminergic neurons (Marongiu et al., 1988; Mayo et al., 1998; Schnell et al., 2003; Millhorn et al., 1997; Johansen et al., 2010; Fujita et al., 1989). They have been widely used *in vitro* to investigate the pathogenesis of neurodegenerative and ischemic neural diseases (Pittenger et al., 1999; Uccelli et al., 2008; Marongiu et al., 1988; Johansen et al., 2010) as well as for drug development (Marongiu et al., 1988; Johansen et al., 2010; Troy et al., 2001; Tabakman et al., 2004; Shimoke and Chiba, 2001; Pan et al., 1997). Our results demonstrated that MDNPC can successfully promote PC12 cell proliferation, neurite formation and dopamine release in a fashion similar to that observed from fetal NS/PC and significantly greater than the ability of parental BMSCs. Furthermore, our study also demonstrated that the mechanism by which MDNPC enhanced PC12 dopamine release correlated with increased expression of the dopamine transporter (DAT). The same mechanism also contributes to the formation of synaptic connections. Therefore, MDNPC may provide as an alternative therapy to NS/PC for the treatment of neuronal diseases.

Materials and methods

Isolation and culture of rat NS/PC and BMSC

NS/PC and BMSC were derived from fetal Sprague Dawley (SD) rat (day 14–16 of pregnancy) brain tissue and adult rat (4–6 weeks old) bone marrow, as previously described (Troy et al., 2001; Tabakman et al., 2004; Gu et al., 2010; Polisetti et al., 2010). This study was evaluated and approved by the Ethical Committee, Fuwai Hospital, Chinese Academy of Medical Sciences. NS/PCs were cultured in Knockout DMEM/F12, supplemented with 2% StemPro® Neural Supplement, 10 ng/ml bFGF, 10 ng/ml EGF, 2 mM L-glutamax, 100 U/ml penicillin, 100 mg/ml streptomycin, and 25 ng/ml amphotericin B (all from Invitrogen). BMSC were cultured and expanded in a complete culture medium containing DMEM-low glucose and 10% fetal bovine serum.

A cell co-culture device was purchased from Millipore Corporation, USA. The culture medium comprised of RPMI1640 + 15% FBS + 2 mM L-glutamax + 100 U/ml penicillin + 100 mg/ml streptomycin and 25 ng/ml Amphotericin B. In experiments involving PC12 cell proliferation, neurite formation, dopamine release and DAT protein expression, the following stages were carried out. First, PC12 cells were collected during the exponential stage and plated in low serum concentration medium (1% horse serum and 3% FBS). The cells were allowed to adhere overnight. The next day the cells were counted and adjusted to a concentration of 2×10^5/ml. Prior to the addition of cells, 400 μl of culture medium was added to the lower part of each well using a 24-well cell culture with insert system. Subsequently, 100 μl of suspended PC12 cells were then added into each well to achieve a cell density of 2×10^4/well, according to preliminary results (data not shown). The MDNPC, NS/PC or MDNPC co-cultures were then seeded at a concentration of 2×10^5/well into the upper chamber (upper insert, $n=3$–5 wells) which were 10 times the number of PC12 cells. The cell cultures were incubated at 5% CO_2, 37 °C for 48 hours. The insert plated cells were then removed and the PC12 cells cultured in the wells were used for subsequent analyses.

Induction of BMSC towards MDNPC

24 hours prior to neural induction, the culture medium was replaced with pre-induction medium containing DMEM, 10% FBS and 1 mM β-mercaptoethanol (BME). To initiate neural precursor cell differentiation, the pre-induction medium was removed. The cells were then washed with PBS and pre-induction medium. An induction medium of DMEM with 1 mM BME was then added for 3 hours (Troy et al., 2001; Woodbury et al., 2000). The induction medium was then replaced with MDNPC maintenance medium which consisted of Knockout DMEM/F12 that contained 2% StemPro® Neural Supplement, 20 ng/ml bFGF, 20 ng/ml EGF, 10 ng/ml leukemia inhibitory factor (LIF) (Millipore), 2 mM L-glutamax, 100 U/ml penicillin, 100 mg/ml streptomycin and 25 ng/ml amphotericin B. Before carrying out subsequent experiments, cells were identified using immunocytochemistry and flow cytometry (Fig. 2). All experiments were carried out using primary cultures (3–5 passages) established from 5–10 rats.

Immunofluorescence staining

Cells were grown on glass coverslips (Solarbio) at 50% confluency for 24 hours. These cells were then fixed with 3.7% paraformaldehyde (Sigma) and permeabilized with 0.02% Triton X-100 (Amresco). Blocking was done with 10% goat serum. Cells were probed with primary antibodies (1:500–1:1000) for 60 minutes, followed by 3 washes and incubation for 1 hour at room temperature with secondary antibodies conjugated to Alexo-488/PE (Invitrogen) (1:2000). Images were captured using a Leica fluorescent microscope (DM2500-3HF-FL1, Germany).

Flow cytometry

To characterize and verify the purity of the rat MDNPC and BMSCs used in this study, the expression of the representative cell markers nestin, integrin β1, CD54 (Pittenger et al., 1999; Pittenger, 2001), fibronectin, type I collagen (Prockop, 1997), CD14 and CD45 (Pittenger et al., 1999; Pittenger, 2001; Alhadlaq and Mao, 2004) (Abcam) was determined using flow cytometry. The cells were dissociated with trypsin–EDTA and re-suspended to a cell concentration of 10^6 cells/ml. The cells were fixed with 3.7% paraformaldehyde with/without permeabilization by 0.02% Triton X-100. Cells were blocked using 5% BSA for 2 hours and the cells were probed with primary antibodies, anti-nestin (1:500) , integrin β1 (1:500), CD54 (1:100), fibronectin (1:1000), type I collagen (1:500), CD14 (1:1000) and CD45 (1:100) (Abcam) for 1 hour at 4 °C. The cells were then rinsed and incubated with anti-rabbit or mouse secondary antibodies conjugated to PE (eBioscience) for 45 minutes, washed and fixed with 2% paraformaldehyde. Data were acquired from 10,000 cells using FACS Calibur and analyzed using Cell Quest-Pro software (BD Biosciences).

BrdU incorporation assay

PC12 cells were seeded at a cell concentration of 5×10^4/well in a 24-well plate and co-cultured with BMSC, NS/PC and MDNPC (5×10^5 500 μl/well) for 48 hours in triplicate. During the last 8 hours, 10 μM of bromodeoxyuridine (BrdU) (Millipore) was added to the culture medium. The cells were then fixed in 4% paraformaldehyde for 20 minutes. After washing, DNA was denatured in 2 M HCl for 30 minutes followed by neutralization in 0.1 M sodium borate for 15 minutes. The cells were washed and permeabilized with 20% normal goat serum and 0.4% triton X-100 (400 μl dissolved in 100 ml of PBS) for 30 minutes to block nonspecific protein binding. The cells were then treated with a monoclonal anti-BrdU antibody (1:200, Millipore) at 37 °C for 1 hour, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rat IgG secondary antibody (1:1000, Millipore) for 30 minutes. 3,3′,5,5′-Tetramethyl benzidine (TMB) peroxidase substrate was added and incubated for 30 minutes at room temperature in the dark. The reaction was halted and the proliferation rates of PC12 cells was quantified using an Infinite™ 200 series Microplate Reader at a dual wavelength of 450/550 nm.

Neurite outgrowth and quantification

Prior to the start of the assay, PC12 cells were cultured for 2–3 days until 60-70% confluent and pre-stimulated for 3 days in differentiation

medium containing nerve growth factor (NGF) (100 ng/ml with 1% horse serum, 3% FBS). The morphology of the PC12 cells was observed using a microscope. The neurite outgrowth assay was performed according to the manufacturer's protocol (NS225, Millipore). PC12 cells allowed to extend their neurites for 24 hours at 37 °C on the insert membrane, which was coated with 10 μg/ml collagen or 2 mg/ml BSA, while MDNPC, BMSC and NS/PCs were grown on the bottom of the 24 well plates. PC12 cells were grown at a density of 5×10^4 in 500 μl of media per well. After washing, fixing and staining the neurites with Neurite Stain Solution (Part NO 90243, Millipore), cells were visualized for morphological changes under an inverted phase contrast microscope (Leica, USA) and images were captured. For quantification, the cell bodies of PC12 cells were removed from the surface of the insert membranes with a cotton swab. A 100–200 μl drop of neurite stain extraction buffer (Neurite Stain and Extraction Buffer, Millipore, USA) was placed onto a flat piece of parafilm and the underside of the insert membrane that contained the stained neurites was positioned onto the drop of extraction buffer. The underside of the insert membrane was incubated with extraction buffer for five minutes at room temperature. Thereafter, 100–200 μl of extraction buffer (only containing outgrown neurites) was collected and measured using a spectrophotometer at 562 nm, as a means of quantifying the extent of neurite outgrowth in each experimental group.

Dopamine release assay

To detect the release of dopamine from PC12 derived neural cells induced by BMSC, NS/PC, MDNPC and NGF, media were collected from cultures grown as previously described in the neurite outgrowth assays. Solid phase enzyme-linked immunosorbent assay (ELISA) was then performed according to procedures suggested by the manufacturer (40-371-25013; GenWay Biotech). The antibody was diluted 500-times using packet buffer and left overnight at 4 °C. On the second day, the plate was washed thoroughly and the diluted standard control and samples were added and incubated at 37 C for 1 hour. This was carried out similarly for negative controls. Horseradish peroxidase was added into each well for incubation for 0.5–1 hour at 37 °C then 0.1 ml of the freshly prepared TMB substrate was added and incubated at 37 °C for 10–30 minutes. Approximately 2 M sulfuric acid was used to stop the reaction. The OD values were measured at 405 nm using an Infinite™ 200 series Microplate Reader after setting the blank control to 0. Finally the dopamine content in the samples was determined from the standard curve.

SDS–PAGE and Western blot

To determine DAT expression levels, proteins were isolated from neuron-like PC12 cells obtained from the previous PC12 co-culture. PC12 cells were collected, washed with ice-cold PBS (pH 7.4) and re-suspended in pre-cooled lysates (containing 1% NP-40, 0.5%sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 33 μg/ml Aprotinin and 1 mmol/l Sodium orthovanadate pH7.4). The mixture was incubated at 4 °C for 30 min and followed by centrifugation at 4 °C, 12,000*g* for 10 min. The protein concentration of the supernatant was determined using the BCA determination kit (Solarbio, Peking, China). Respectively, the same amount of protein of each set of parallel holes were mixed with the same volume of 2× SDS buffer and boiled for 5 min. Ten microliters of protein samples were separated using 10% SDS–polyacrylamide gels and transferred onto polyvinylidene fluoride (PVDF) membranes. The PVDF membranes were blocked with 5% non-fat dried milk in Tris-buffered saline containing 0.1% Tween-20 and probed with primary antibody against 0.2 μg/ml DAT (Abcam) for 2 hours at room temperature. The blots were incubated for 1 hour with HRP-conjugated secondary IgG and immunoreactive bands were detected using SuperSignal Femto Chemiluminescent detection reagent (Pierce, Rockford, IL).

Data analysis

Student's *t*-test was carried out using SigmaStat software (version 3.5). *P* values less than 0.05 were considered statistically significant.

Results

NS/PC and BMSC characterization

NS/PCs were isolated from fetal rat brains and cultured *in vitro*. These cells possessed the self-renewal and plasticity characteristics of stem cells. As shown in Fig. 1A and C, the diameter of the neurospheres increased to approximately 80 μm (80 μm ± 25 μm) after 1 week. The diameter of the neurospheres at day three differed significantly from that at day seven ($P<0.05$) (Fig. 1A). However, the numbers of neurospheres at days three and day seven were not significantly different ($P>0.05$). (Fig. 1B) Immunocytochemistry staining demonstrated that the NS/PCs were positive for the stem cell marker, nestin (Fig. 1C). To identify the plasticity of the NS/PC, these cells were induced to differentiate by culturing in medium containing 10% FBS for 24 hours. We observed positive immunostaining for β-tubulin III (22% ± 3%), GFAP (70% ± 5%) and Gal C (8% ± 2%) which identified neurons, astrocytes and oligodendrocytes, respectively (Fig. 1D).

BMSC were isolated from the bone marrow obtained from the femurs of adult rats (Azizi et al., 1998) and had typical phenotypes and morphological features of bone marrow stromal cells. Flow cytometry analysis of BMSC (passage 3), demonstrated that these cells were positive for cell-surface molecules such as integrin β1, CD54 and the two extracellular matrix molecules, fibronectin and type I collagen (Fig. 1Eiii–vi), that are synthesized by cultured mesenchymal stem cells. Two specific hematopoietic cell surface markers, CD14, which is present on leukocytes and, CD45, which is expressed on monocytes and macrophage, were not detected on these cells (Jiang et al., 2003; Polisetti et al., 2010; Pittenger, 2001) (Fig. 1Ei–ii). As depicted in Fig. 1E, the purity of the BMSC was almost 100%. At low plating densities, the BMSC grew as a monolayer of large, flat cells. As the cells approached confluency, they acquired a more spindle-shaped, fibroblastic morphology (data not shown).

Neural induction of BMSCs

Twenty-four hours prior to neural induction, BMSC were maintained as sub-confluent cultures in serum-containing medium supplemented with 1 mM BME. To initiate neural differentiation, the cells were transferred into serum-free induction medium containing 1 mM BME for 3 hours then, the induction medium was replaced with MDNPC maintenance medium until subsequent experiments. At passage 3, changes in the morphology of the BMSC were apparent and consistent with the findings reported by Sanchez-Ramos J et al. Cell bodies became increasingly spherical, refractile and progressively assumed neural morphological characteristics (Fig. 2A) and approximately 96% of the cells expressed nestin (Fig. 2B).

Proliferation rates of PC12 derived neural cells after co-culturing with BMSC, NS/PC, and MDNPC

Next, we quantified the proliferation rates of PC12 cells induced by co-culturing with MDNPC, NS/PC and BMSC. PC12 cells were first incubated in medium with a low concentration of serum (1% horse serum and 3% fetal bovine serum) for 24 hours. As calculated from the cell number curve (Fig. 3A), 5×10^4 PC12 cells were seeded into each well of a 24-well plate and co-cultured with each of the following cell types, BMSC, NS/PC, and MDNPC at a cell concentration of 5×10^5/well for 48 hours (based on preliminary results by adding 10 times the number of effector cells). Approximately 100 ng/ml of NGF treatment was also incubated with the PC12 cells as a positive

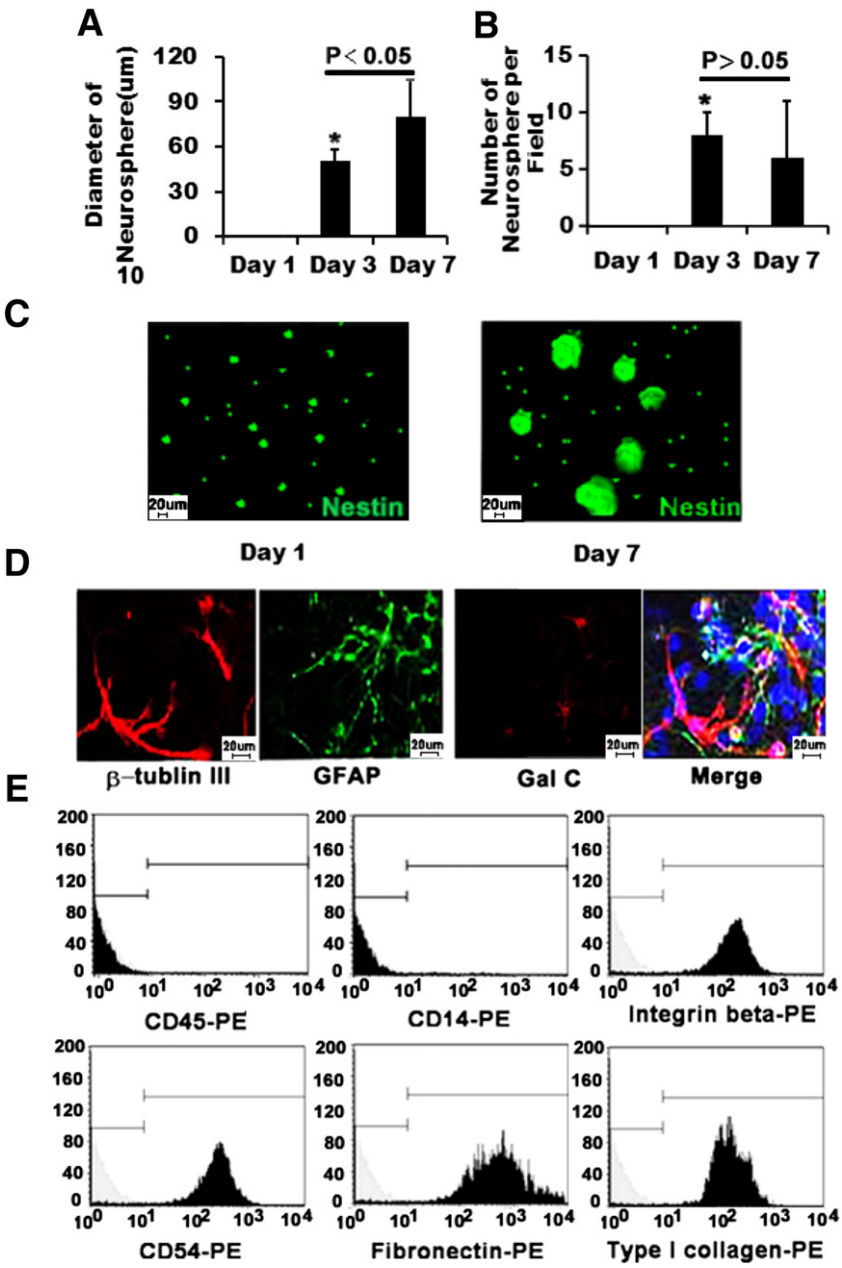

Fig. 1. Characterization of NS/PC and BMSC. (A)–(D) Self-renewal and plasticity properties of NS/PC. Fetal rat NS/PCs were cultured *in-vitro* for 1, 3 and 7 days, the diameters (A) and numbers (B) of neurospheres were counted, then the cells were immunostained and photomicrographs were taken using a Leica fluorescent microscope (C). NS/PC neurospheres stained positive for nestin (Green fluorescence, Alexa 488). (D) After the differentiation of NS/PC in medium containing 10% FBS for 24 hours, the cells were assessed using immunostaining. These differentiated NS/PC were positive for the neural protein β-tubulin III (red), the astrocyte protein GFAP (green) and the oligodendrocyte protein Gal C (red). Neurospheres with diameters greater than 20 μm in 10 visual fields were counted using the microscope. Following which, the mean of the cell number and diameter were taken into account. Scale bar = 20 μm. (E) Characterization of BMSC before differentiation. Flow cytometry was carried out on passage 3 BMSC. Monoclonal antibodies were directed against cell surface markers integrin β1, CD54, fibronectin, type I collagen, CD14 and CD45. PE-conjugated goat anti-rabbit antibody was used as the secondary antibody. Dark shaded histograms depict cells which are positive for PE labeled CD54, integrin β11, fibronectin and type I collagen. The light gray shaded histograms depict isotype controls. The above histograms are representative of five independent experiments. $^{*}P<0.05$, day 3 vs. day 7 in Fig. 1A; $^{*}P>0.05$, day 3 vs. day 7 in Fig. 1B.

control and co-culture without treatment served as the no treatment control. A BrdU incorporation assay was subsequently performed as described in the Materials and methods. As shown in Fig. 3B, co-culturing PC12 cells with NS/PC and MDNPC, as well as treatment with NGF (positive control) induced a significant increase in proliferation rate in PC12 cells as compared to PC12 cells co-cultured with BMSC or negative control ($^{*}P<0.05$ vs. MDNPC group). Proliferation rates of PC12 cells that were co-cultured with NS/PC, MDNPC or treated with NGF were not significant. ($^{**}P>0.05$ vs. MDNPC group, respectively).

Neurite outgrowth of PC12 cells

When observing neurite outgrowth formation, it was found that sprouting of neurite-like extensions was induced from PC12 cells that were co-cultured with NGF, NS/PC and MDNPC, after 24 hours of incubation on a collagen coated surface (Fig. 4A). However, this phenomenon was not observed in cells co-cultured with BMSC, cells grown on a BSA-coated surface or cells with no treatment/negative controls (Fig. 4A).

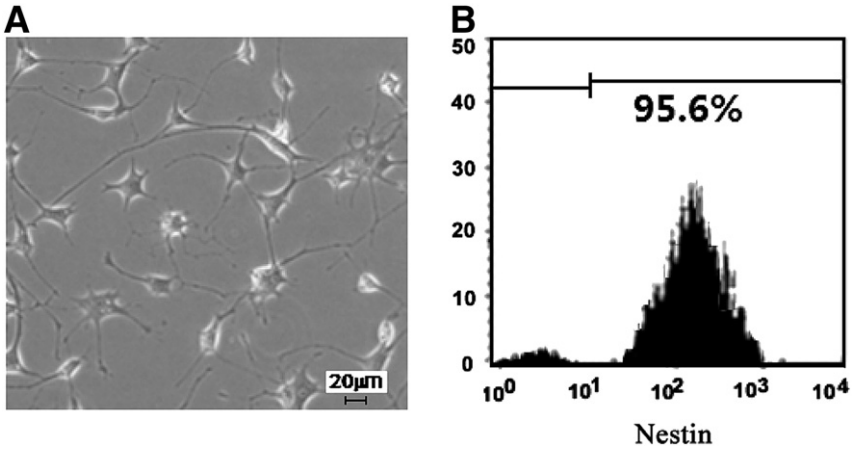

Fig. 2. Characterization of MDNPCs derived from BMSCs. (A) The morphology of MDNPC. BMSC were differentiated into MDNPC after several hours of exposure to BME and cultured in neural progenitor cell maintenance medium. At passage 3, photomicrographs were taken using a phase contrast inverted microscope. Scale bar = 20 μm. (B) The purity of MDNPC. After MDNPC were expanded in stem cell maintenance medium, at passage 3, cells were stained with primary antibody against rat nestin and PE-labeled secondary antibody to measure nestin positive MDNPC.

Next, quantification of the magnitude of neurite outgrowth from PC12 cells co-cultured with the different experimental groups, BMSC, NS/PC and MDNPC was determined. The extraction buffer (only containing outgrown neurites) was collected and measured using a spectrophotometer at 562 nm, as a means of quantifying the extent of neurite outgrowth in each experimental group. As compared with the untreated PC12 cells, the BSA coated or BMSC co-cultured groups, the PC12 cells that were co-cultured with NS/PC and MDNPC displayed a significant difference in the quantity of neurite outgrowth with long neurite-like cytoplasmic extensions. ($P<0.05$, NS/PC and MDNPC group vs. PC12 alone, BSA coated or BMSC treated group). With an absorbance (OD) ratio of 1, the quantity of PC12 neurite outgrowth in the MDNPC co-cultured group was similar to that of the NS/PC co-cultured group ($P>0.05$, MDNPC group vs. NS/PC group) (Fig. 4B).

Dopamine release and the expression of DAT in PC12 derived neural cells

In order to compare the effects of the different types of stem cells on dopaminergic transmitter release from PC12 cells, we determined the dopamine concentration present in the supernatant of PC12 derived neural cells when co-cultured with NGF, BMSC, NS/PC and MDNPC using ELISA. This assay demonstrated that MDNPC stimulated PC12 derived neural cells to produce comparable levels of dopamine (an average of 3.48 fold increase compared with control), when compared with NS/PC (an average of 3.52 fold increase compared with control) ($^{*}P>0.05$). However, there was a significant difference in the levels of dopamine released when compared to the NGF (an average of 1.08 fold increase compared with control) treated group or the non-transdifferentiated BMSC co-culture (an average of 1.5 fold increase compared with control) treated group ($^{**}P<0.05$) (Fig. 5A). These observations indicated that the effect of regulating dopamine release from MDNPC co-culture was superior to the BMSC or NGF co-culture and shared similar effects with the NS/PC co-culture.

DAT is expressed on dopaminergic neurites and it regulates the balance between dopamine up-take and release. To provide further evidence to demonstrate the regulatory effect of stem cell secretions on dopamine release, we examined the expression of DAT by PC12 derived neural cells after co-culture with MDNPC, NS/PC, BMSC or NGF treatment using western blot. As shown in Fig. 5B, using gel image analysis software (Sigma Scan Pro. SPSS, Inc. Chicago, IL, USA) we observed

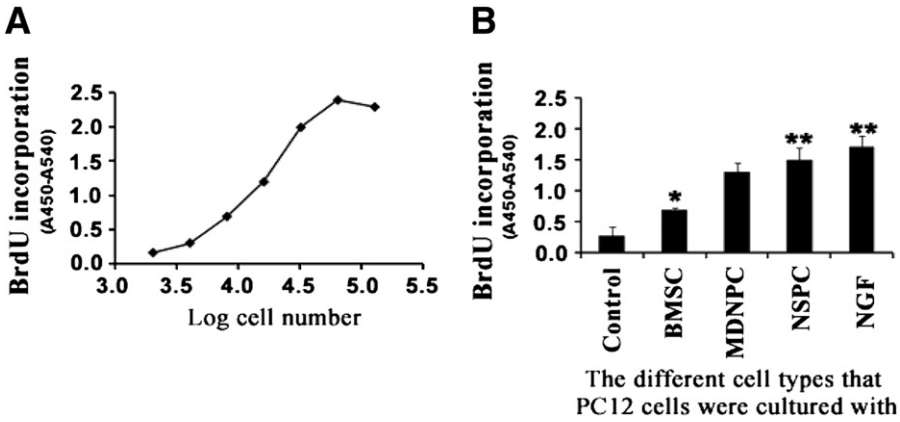

Fig. 3. The proliferation rates of PC12 cells induced by culturing with MDNPC, NS/PC and BMSC. (**A**) A cell number curve was used to determine the optimal number of PC12 cells required for seeding into each well of a 24-well plate, so that the absorbance levels at 450/550 nm falls within the instruments measurement limits. (B) After resting for 24 hours in medium with low concentration of serum (1% horse serum and 3% fetal bovine serum), PC12 cells (5×10^4/well) were co-cultured with each of the following cell types, BMSC, NS/PC and MDNPC at a cell concentration of 5×10^5/well, for 48 hours. NGF treatment was also incubated with the PC12 cells as a positive control. BrdU was added during the last 8 hours and the BrdU incorporation assay was carried out as described in Materials and methods. The proliferation rates of PC12 derived neural cells in the different groups of co-culture were measured and depicted as bar graphs. $^{*}P<0.05$ vs. MDNPC group; $^{**}P>0.05$ vs. MDNPC group for both, paired *t*-test. Results were obtained from three independent experiments.

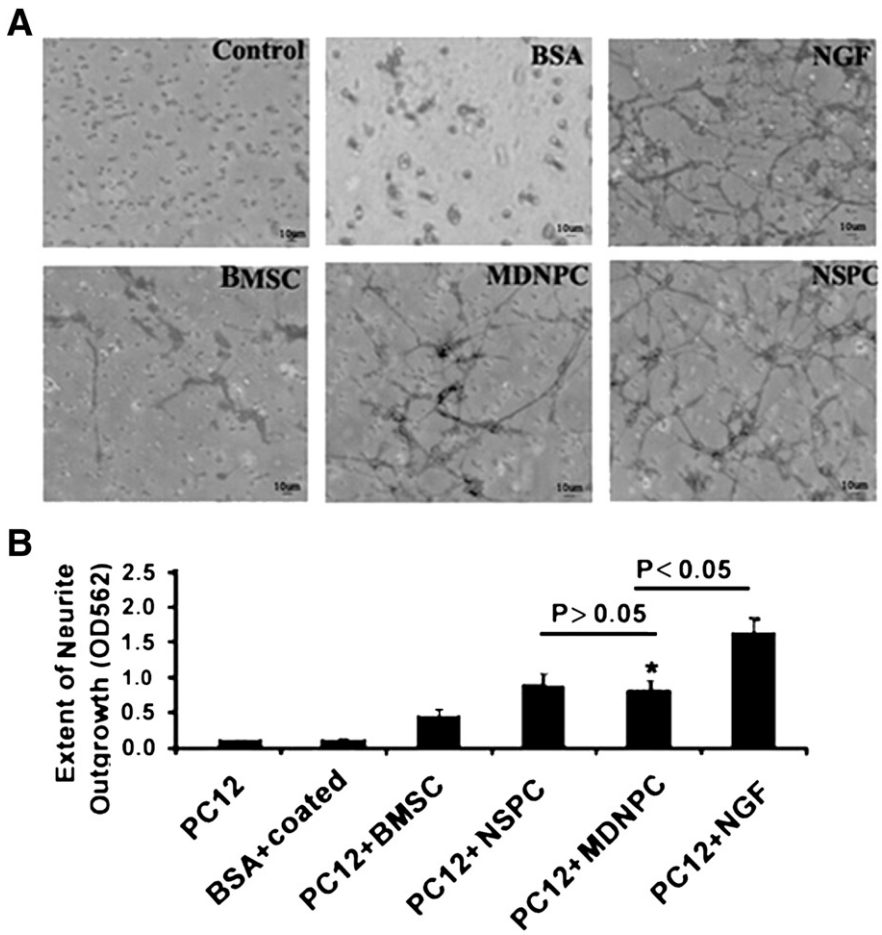

Fig. 4. Neurite outgrowth in PC12 cells induced by co-culturing with MDNPC, NS/PC, MSC and NGF treatment. (A) 1×10^4/well of PC12 cells were allowed to extend their neurites for 24 hours at 37 °C on the insert membrane which was coated with 10 μg/ml collagen, while MDNPC, BMSC and NS/PCs were grown at the bottom of the 24 well plate. PC12 cells were also grown in the presence of 100 ng/ml of NGF to serve as a positive control and also on a BSA-coated surface to serve as a negative control. After washing, fixing and staining the neurites with Neurite Stain Solution, the cells were visualized for morphological changes under an inverted phase contrast microscope and photos were taken. Scale bar = 10 μm (upper panel). **(B)** The quantification of neurite outgrowth from PC12 derived neural cells in each experimental group was carried out. Cell bodies were removed from the surface of insert membrane (as described in detail in Materials and methods) and the stained outgrown neurites were extracted using Neurite Stain Extraction Buffer. The extraction buffer was then collected and measured using a spectrophotometer (562 nm), as a means of quantifying the extent of neurite outgrowth in each experimental group. $^{*}P<0.05$, vs. PC12 alone, BSA coated, BMSC or NGF treated group; $^{*}P>0.05$ vs. NS/PC treated group, Student's *t*-test. Results were obtained from at least three independent experiments.

that PC12 derived neural cells from the co-cultured NS/PC and MDNPC treatment groups demonstrated higher levels of DAT expression, whereas the BMSC and NGF co-cultured experimental groups demonstrated lower levels of DAT expression ($^{**}P<0.05$ vs. MDNPC group, respectively). Comparable levels of DAT expression were detected between the MDNPC and NS/PC co-cultured groups ($^{*}P>0.05$). These relative

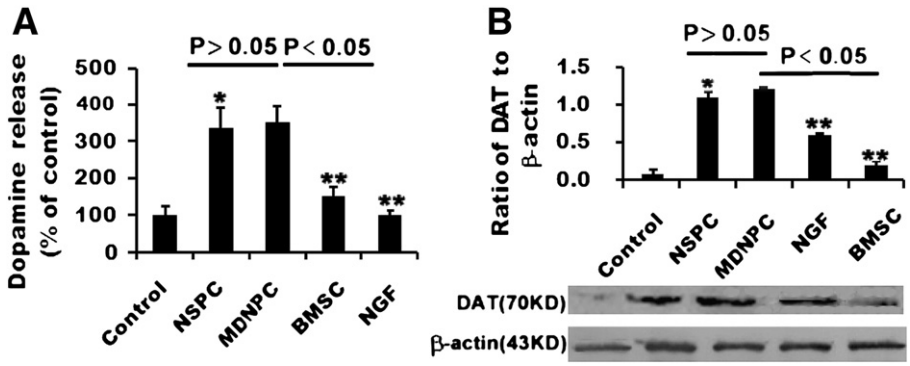

Fig. 5. Measurement of dopamine release and DAT expression levels from PC12 derived neural cells. (A) To compare the effects on dopamine release, PC12 derived neural cells were co-cultured with BMSC, NS/PC, MDNPC or NGF treated. After 48 hours, levels of dopamine in cell culture supernatants were measured using ELISA. $^{*}P>0.05$ and $^{**}P<0.05$, compared with the MDNPC co-culture group. (B) Western blot analysis of cellular lysates was carried out to examine protein levels of DAT (MW = 70 kDa) expressed by PC12 derived neural cells co-cultured with BMSC, NS/PC, MDNPC or NGF treated. Lysates were blotted with beta actin as a loading control. Using the Sigma Scan Pro. SPSS gel image analysis software, the ratio of DAT to beta-actin was determined. $^{*}P>0.05$ and $^{**}P<0.05$, compared with the MDNPC co-culture group. Results were obtained from at least three independent experiments.

differences in DAT protein levels exhibited a similar profile to the dopamine release pattern for each of the experimental treatment groups. Taken together, these data demonstrate that the expression pattern of DAT in PC12 derived neural cells closely correlates with the release of dopamine.

Discussion

Many pre-clinical and clinical trials using stem cells from various sources to treat intractable neural system diseases have been reported (Goldman, 2005). The functions of stem cells or progenitors in neural regeneration involve complex processes including axonal regeneration, remyelination, and the formation of new synaptic connections, which depend on the release of neurotransmitters. In this study, we investigated the neural regenerative effects of MDNPC from adult rat bone marrow in terms of several key points in neural regeneration, which include proliferation, neurite formation and dopamine neurotransmitter release in PC12 derived neural cells. We also explored the possibility that MDNPC can be used as an alternative cell source besides NS/PC for the treatment of CNS diseases. As animal models can simulate some of the pathological features of disease, but do not reflect both anatomical and molecular characterization of all diseases (Pittenger, 2001; Phinney and Prockop, 2007), cell culture models are increasingly being utilized in research. We have chosen PC12 cells (Koch et al., 2009) in this study because they have been shown to be a reliable dopaminergic neuron model (Marongiu et al., 1988; Mayo et al., 1998; Schnell et al., 2003; Millhorn et al., 1997; Johansen et al., 2010; Fujita et al., 1989) and have been widely used to investigate the pathogenesis of neurodegenerative and ischemic neural system diseases (Troy et al., 2001; Tabakman et al., 2004; Shimoke and Chiba, 2001; Pan et al., 1997) at a cellular and molecular level as well as in drug development studies. Nevertheless, we appreciate the caveat of these cells being a tumorigenic line and suggest that future follow-up studies be performed in primary cells to fully validate our conclusions drawn from the PC12 line.

Transdifferentiation is a newly emerging concept, which is defined as the differentiation of a tissue-specific stem cell into another cell type. For example, bone marrow MSC can transdifferentiate into neurons (Jiang et al., 2003; Black and Woodbury, 2001; Pittenger et al., 1999; Uccelli et al., 2008; Kopen et al., 1999). J. Sanchez-Ramos et al. (2000) found that during the transdifferentiation of BMSC into neurons, the majority of cells expressed the neural stem cell specific marker (nestin) within the first 5 hours. This indicates that nestin positive MDNPC are an essential intermediate product present prior to neuron derivation. Therefore, we stopped the transdifferentiation process after 3 hours and then cultured them in serum free maintenance medium containing Knockout DMEM/F12, StemPro Neural supplement, bFGF and EGF to maintain their neural stem cell phenotype. The presence of LIF prevents MDNPC differentiation towards a terminally differentiated cell. Furthermore, 95% of the MDNPC population remained nestin positive at passage 3, confirming the possibility of using MDNPC as an alternative cell source to NS/PC for the treatment of CNS diseases

Xu et al. (2010) and others have illustrated that MDNPC could improve neurological function after gerbil stroke, although the mechanism remains unclear (Hayase et al., 2009). Our current investigation is the first to compare the effects of MDNPC, NS/PC and NGF on neural regeneration. In our study, we demonstrated that adult MDNPC behave similarly to fetal NS/PC. The contribution of neural regeneration by MDNPC, occurs not only via promoting proliferation and neurite outgrowth of neural cells (which has the same effects as NGF treatment), but also by modulating dopamine release from PC12 cells *in vitro*, an effect which was even higher than NGF treatments. Many studies have confirmed that BMSC can effectively alleviate or even repair nerve damage. However, in our study, while MDNPC and NSP/C showed a significant role in promoting PC12 cell proliferation and neurite formation, BMSC did not stimulate statistically significant effects when compared to the control group. Whether this phenomenon is related to the transdifferentiation of BMSC, needs further investigation. The molecular mechanisms underlying MDNPC-induced dopamine release have not yet been reported. Our results suggest that the enhanced dopamine release is associated with the upregulation of DAT expression. We also determined that the effects of MDNPC on neural regeneration were greater than those induced by parental BMSC. One reason for this difference may be that BMSC are heterogeneous with multipotent capabilities, while MDNPC are derived specifically from the neural system and hence more specialized.

DAT is a membrane-spanning protein that controls the concentration of dopamine within synapses. DAT removes dopamine from the synaptic cleft and deposits it into surrounding cells, thus terminating the dopamine signaling pathway. On the other hand, when the neuron depolarizes, DAT induces dopamine release (Wheeler et al., 1993; Schultz, 1998; Brooks, 2010). Therefore, DAT activity may have the same effects as a drug treatment that increases dopamine levels in patients with neurodegenerative diseases, ischemic or hemorrhagic stroke and spinal cord injuries (Ahrlund-Richter et al., 2009). The bi-directional transport function of DAT can also explain why the expression levels of DAT in our study were not in direct correlation with the levels of dopamine release induced by MDNPC, NS/PC, BMSC and NGF (Fig. 4).

Conclusion

In summary, to the best of our knowledge, this is the first study to provide evidence that MDNPC play a role comparable to NS/PC in inducing neurite outgrowth and dopamine release in a commonly used neural system model, PC12 cells. Moreover, dopamine release induced by stem cells was related to changes in DAT levels. MDNPC may be a promising candidate source of neural stem cells for clinical applications. However, MDNPC, like all other types of stem cells, have the potential to generate tumors (Fong et al., 2010), and must be carefully assessed in animals experiments prior to use in clinical applications.

Conflict of interest statement

The authors declared no conflict of interest.

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