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Neurogenic and cardiomyogenic differentiation of mesenchymal stem cells isolated from minipig bone marrow

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ABSTRACT

The present study investigated the potential of minipig bone marrow-mesenchymal stem cells (BM-MSCs) to differentiate *in vitro* into neuron- and cardiomyocyte-like cells. Isolated BM-MSCs exhibited a fibroblast-like morphology, expressed CD29, CD44 and CD90, and differentiated into osteocytes, adipocytes and chondrocytes. Upon induction in two different neuronal specific media, most of BM-MSCs acquired the distinctive morphological features and positively stained for nestin, neurofilament-M (NF-M), neuronal nuclei (NeuN), β-tubulin, galactocerebroside (Gal-C) and glial fibrillary acidic protein (GFAP). Expression of nestin, GFAP and NF-M was further demonstrated by RT-PCR and RT-qPCR. Following cardiomyogenic induction, MSCs exhibited a stick-like morphology with extended cytoplasmic processes, and formed cluster-like structures. The expression of cardiac specific markers α-smooth muscle actin, cardiac troponin T, desmin and α-cardiac actin was positive for immunofluorescence staining, and further confirmed by RT-PCR and RT-qPCR. In conclusion, our results showed the *in vitro* differentiation ability of porcine BM-MSCs into neuron-like and cardiomyocyte-like cells.

1. Introduction

The similarities in basic biological properties of mesenchymal stem cells (MSCs) between porcine and human make porcine MSCs as a highly relevant cell source to optimize methods for the purity of isolation, specific identification, culture expansion and *in vitro* characterization for cell based therapeutic strategies (Rho et al., 2009). Recently, the use of minipigs has gained considerable importance in various fields of biomedical research including the assessment of MSCs applications. This is mainly due to their large similarities in organ physiology, morphology, development and genetic characteristics with humans, and thus allowing the findings acquired from other models to be validated and further assessed for safety and efficiency before bringing the cell therapy into reality (Fu et al., 2006; Juhásová et al., 2011; Park et al., 2011).

In pigs, MSCs can be successfully isolated from various tissues (Ringe et al., 2002; Mitchell et al., 2003; Faast et al., 2006; Huang et al., 2007; Kumar et al., 2007; Ock et al., 2010; Park et al., 2011) and induced to differentiate *in vitro* into osteocytes, adipocytes, chondrocytes (Ringe et al., 2002; Vacanti et al., 2005; Faast et al., 2006; Huang et al., 2007; Kumar et al., 2007), neuronal cells (Mitchell et al., 2003; Weiss et al., 2003; Huang et al., 2007), smooth muscle cells (Shukla et al., 2008), myocytes/cardiomyocytes (Tomita et al., 2002; Liu et al., 2004; Moscoso et al., 2005) and other cell types (Fu et al., 2006). Porcine MSCs from different tissue origins have showed the expression of a panel of immunophenotypic markers and largely identical *in vitro* expansion characteristics. Furthermore, by virtue of broader differentiation ability into distinctive cells, porcine MSCs offer a great value to accomplish substantial advances in regenerative medicine and tissue engineering. Despite some significant progress in demonstrating the differentiation capabilities of MSCs across mesenchymal tissue lineages, the evidences on their ability to transdifferentiate *in vitro* into cells different from their tissue origin requires further assessment (Rho et al., 2009). In view of this, it emphasizes the need of *in vitro* differentiation studies in porcine model for evaluating the molecular and functional mechanisms of MSCs plasticity.

The potential of post-natal minipig bone marrow-mesenchymal stem cells (BM-MSCs) to acquire a neuronal phenotype *in vitro* has not been examined. Furthermore, MSCs from bone marrow have been proposed as useful candidates in mediating myocardial regeneration owing to their unique properties (Tomita et al., 2002). Therefore, the main purpose of this study was to investigate

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the ability of minipig BM-MSCs to differentiate *in vitro* into neuron-like cells by employing two different neural induction methods and cardiomyocyte-like cells by chemical stimulation for different exposure times. Isolated BM-MSCs showed spindle-shaped morphology, expressed mesenchymal cell specific surface markers and differentiated into osteocytes, adipocytes and chondrocytes. Neuronal and cardiomyogenic differentiation were characterized by the distinctive morphological features and the expression of specific markers at protein and mRNA levels. Our results demonstrated that BM-MSCs exhibit broader potentialities apart from differentiating into cell types of bone, fat and cartilage.

2. Materials and methods

All experimental procedures were conducted in accordance with the Animal Center for Biomedical Experimentation at Gyeongsang National University.

2.1. Chemicals and media

All chemicals were purchased from Sigma chemical company (Sigma, St. Louis, MO, USA) and media from Gibco (Invitrogen, Burlington, ON, Canada), unless otherwise specified.

2.2. Isolation and culture of minipig bone marrow MSCs

MSCs were isolated from bone marrow extract of a post-natal female miniature piglet [T-type, PWG Micro-pig (R), PWG Genetics Korea], immediately after birth as previously described (Vacanti et al., 2005). Briefly, bone marrow extract was diluted with Dulbecco's phosphate buffered saline (D-PBS) solution and layered on Ficoll-Paque™ Plus (1.077 g/ml, GE Healthcare Bioscience AB, Sweden) following which the sample was centrifuged at 400*g* for 40 min. The interface fraction containing the mononuclear cells was washed twice with D-PBS and cultured in advanced Dulbecco's modified Eagle's medium (ADMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 IU/ml penicillin at 38.5 °C in a humidified atmosphere of 5% CO_2 in air. Once confluent, cells were dissociated using 0.25% trypsin–EDTA solution and pelleted at 500*g* for 5 min. Cells were cryopreserved or re-propagated for further analysis. All experiments were set up within passage 6 of MSCs.

2.3. Cell surface antigen profile by flow cytometry

Bone marrow derived cells were characterized for the presence of mesenchymal markers (CD29, CD44, CD90) and absence of CD34, CD45 and major histocompatibility complex II (MHCII) using flow cytometry (BD FACSCalibur, Becton Dickinson, NJ, USA) before performing different experiments. Cells at ~80% confluence were fixed with 3.7% formaldehyde for 30 min. Fluorescein isothiocyanate (FITC) conjugated mouse anti-CD44, CD90 and CD34 (BD Pharmingen™, Becton Dickinson) were labeled directly at 4 °C for 1 h, and unconjugated CD29 (BD Pharmingen™), CD45 and human MHCII (Santa Cruz biotechnology, CA, USA) were incubated for 45 min at 37 °C. Following D-PBS washes, FITC-conjugated goat anti-mouse IgG (BD Pharmingen™) used as a secondary antibody was labeled for 1 h at 4 °C. The standard was established by isotype-matched control. A total of 10,000 FITC-labeled cells were acquired and analyzed by a BD FACSCalibur with CellQuest software (Becton Dickinson).

2.4. In vitro differentiation into osteo, adipo and chondrocytes, and cytochemical staining

By following previously published protocols with minor modifications (Pittenger et al., 1999; Vacanti et al., 2005), BM-MSCs were cultured in ADMEM containing 10% FBS under osteogenic, adipogenic and chondrogenic conditions for 21 days (media change was done every 3 or 4 days) to evaluate their mesenchymal differentiation potential. Untreated cells were maintained in ADMEM with 10% FBS.

Osteogenic inductive media contained 1 μM dexamethasone, 10 mM sodium β-glycerophosphate and 0.05 mM ascorbic acid. At 12 days of induction, the cells were stained with BCIP/NBT (Promega, Madison, WI, USA) to assess alkaline phosphatase (AP) activity. Mineralization was detected by von Kossa staining after 21 days.

Adipogenic inductive media contained 10 μM insulin, 100 μM indomethacin, 500 μM isobutyl methylxanthine, and 1 μM dexamethasone. Oil droplets in differentiated adipocytes were evaluated by Oil red O staining after 21 days.

Chondrogenic differentiation was induced in confluent monolayer cultures with 5 ng/ml transforming growth factor-β1 (TGF-β1, R&D systems, Minneapolis, MN, USA), 0.1 μM dexamethasone, 50 mg/ml ascorbic acid, 100 mg/ml sodium pyruvate, 40 mg/ml L-proline and 50 mg/ml ITS^+ premix (6.25 mg/ml insulin, 6.25 mg/ml transferrin, 6.25 ng/ml selenious acid, 1.25 mg/ml BSA and 5.35 mg/ml linoleic acid). Proteoglycan deposition was assessed by Alcian blue 8GX solution after 21 days.

2.5. Neurogenic differentiation

Neuronal differentiation was induced by two different protocols with minor modifications. In Woodbury et al. (2000) protocol, BM-MSCs at 70% confluence were pre-incubated in ADMEM supplemented with 20% FBS, 1 mM β-mercaptoethanol (BME) and 10 ng/ml basic fibroblast growth factor (bFGF) for 24 h. The cells were then cultured in ADMEM supplemented with 2% dimethyl sulfoxide (DMSO), 200 μM butyrated hydroxyanisole (BHA), 2 mM valproic acid, 10 μM forskolin, 5 μg/ml insulin, 1 μM hydrocortisone and 25 mM KCl for 6 days, and this method was designated as BME/DMSO/BHA neural induction. Another protocol was adapted from Chao et al. (2007) and to explain briefly, BM-MSCs at 80% confluence were cultured in ADMEM supplemented with 10% FBS, 20 ng/ml epidermal growth factor (EGF) and 10 ng/ml bFGF for 24 h before it was replaced with 30 μM trans-retinoic acid (RA). The cultures were maintained for 10 days and the medium containing RA was replaced every 3 or 4 days. This protocol was designated as RA neural induction. In both methods, uninduced cells were maintained in ADMEM with 10% FBS.

2.6. Cardiomyogenic differentiation

The differentiation of cardiomyocyte-like cells from BM-MSCs was performed as previously described (Tomita et al., 2002; Zhang et al., 2009) with minor modifications. Briefly, BM-MSCs at 60% confluence were treated with 10 μM 5-azacytidine (5-azaC) for either 24 h, 3 days or 7 days. Cells were cultured in ADMEM containing 10% FBS after the drug treatment. All treatments were terminated at day 21 of total culture, with the medium changes every 3 or 4 days. Untreated cells were maintained in ADMEM with 10% FBS.

2.7. Immunofluorescence analysis

Expression of neuronal and cardiomyocyte specific markers were analyzed by immunofluorescence staining. Induced and uninduced BM-MSCs were washed twice with D-PBS before being fixed