Journal of Proteomics xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Proteomic analysis of mesenchymal to Schwann cell transdifferentiation

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ARTICLE INFO

Article history:
Received 18 April 2017
Received in revised form 31 May 2017
Accepted 13 June 2017
Available online xxxx

Keywords:
Schwann cells
Axon regeneration
Nerve growth factor
Brain-derived neurotrophic factor
Neurite outgrowth
Remyelination
Mesenchymal stem cells
Transdifferentiation
Proteomics
Systems biology
Peripheral nerve regeneration

ABSTRACT

While transplantation of Schwann cells facilitates axon regeneration, remyelination and repair after peripheral nerve injury clinical use is limited by cell bioavailability. We posit that such limitation in cell access can be overcome by the use of autologous bone-marrow derived mesenchymal stem cells (MSCs). As MSCs can transdifferentiate to Schwann cell-phenotypes and accelerate nerve regeneration we undertook proteomic evaluation of the cells to uncover the protein contents that affects Schwann cell formulation. Transdifferentiated MSCs secrete significant amounts of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in cell-conditioned media that facilitated neurite outgrowth. MSC proteins significantly regulated during Schwann cell transdifferentiation included, but were not limited to, GNAI2, MYL9, ACTN4, ACTN1, ACTB, CAV-1, HSPB1, PHB2, TBB4B, CTGF, TGFI1, ARF6, EZR, GELS, VIM, WNT5A, RTN4, EFNB1. These support axonal guidance, myelination, neural development and neural growth and differentiation. The results unravel the molecular events that underlie cell transdifferentiation that ultimately serve to facilitate nerve regeneration and repair in support of cell transplantation.

Significance statement: While Schwann cells facilitate axon regeneration, remyelination and repair after peripheral nerve injury clinical use is limited by cell bioavailability. We posit that such limitation in cell access can be overcome by the use of bone-marrow derived mesenchymal stem cells (MSCs) transdifferentiated to Schwann cell-phenotypes. In the present study, we undertook the first proteomic evaluation of these transdifferentiated cells to uncover the protein contents that affects Schwann cell formulation. Furthermore, these transdifferentiated MSCs secrete significant amounts of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in cell-conditioned media that facilitated neurite outgrowth. Our results demonstrate that a number of MSC proteins were significantly regulated following transdifferentiation of the MSCs supporting roles in axonal guidance, myelination, neural development and differentiation. The conclusions of the present work unravel the molecular events that underlie cell transdifferentiation that ultimately serve to facilitate nerve regeneration and repair in support of cell transplantation. Our study was the *first proteomic comparison demonstrating the transdifferentiation of MSCs* and these reported results can affect a wide field of stem cell biology, tissue engineering, and proteomics.

1. Introduction

Peripheral nerve injuries result in disruption of neural signaling that occurs between affected limbs and the central nervous system (CNS) often leading to severe neurological morbidities and even paralysis [1]. Anterograde (Wallerian) degeneration initiates a cascade of mixed degenerative and regenerative events initiated by infiltration of peripheral blood derived monocyte-macrophages and end organ Schwann cells (SCs). The former serve to remove the debris created by degradation of axonal tissue, axonal fibers and the myelin sheath [2] and the latter provide cells for regenerative activities. SCs present in the distal end of the injured tissue effect the secretion of neurotrophic factors. Such factors attract axons regenerating from the proximal end of the injury [3]. If sufficient support is provided to the regenerating axonal growth cones, they may navigate across the nerve gap through a nerve bridge and then grow into the remaining skeleton of degenerated neural tissue. In more extreme cases where a large gap may exist, a range of strategies, are operative that provide support for regenerating neural tissue. This is referred to as a "nerve guidance conduit" which can be used to physically bridge the transected ends of the nerve and as such provide

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http://dx.doi.org/10.1016/j.jprot.2017.06.011
1874-3919/

support for regenerating axons [4–7]. Nerve guidance conduits filled with SCs is a promising strategy to promote neural and tissue regeneration following injury [8–10]. However, the limited availability, donor site morbidity, cumbersome isolation and limited *in vitro* growth of SCs restricts their use for transplantation [11,12]. To obviate such limitations, researchers have turned to alternative cellular sources including mesenchymal [13–15], neural [16,17], and olfactory ensheathing stem cells [18,19] amongst other strategies [20,21] to replace lost SCs after nerve injury.

Bone marrow-derived mesenchymal stem cells are one of a few stem cell types that have been successfully applied to the clinic for tissue repair. In addition to their multipotent differentiation capacity they possess a number of advantages. These include, but are not limited to, ease of isolation and maintenance, ability to survive and migrate following transplantation, and in permitting autologous transplantation. These together can circumvent problems associated with an immune response along with precluding any ethical concerns. Mesenchymal stem cells also possess strong paracrine activity proposed as a principal mechanism for tissue repair and can be engineered to produce exogenous therapeutic proteins and transdifferentiate into neural cell types [5]. Such unique abilities make MSCs an invaluable cell type for neural repair. We posit that success with this approach would provide patients opportunities to donate their own stem cells, which could be implanted into nerve guidance conduits or transplanted at the site of injury in order to facilitate nerve repair [22–25].

Mesenchymal stem cells transdifferentiation into Schwann-like cells (referred to tMSCs) and their subsequent incorporation into nerve regeneration conduits promotes axonal regeneration, reduces lesion size, enhances neuronal survival and improves functional outcomes [14,15,26–36]. Although the success of cell transdifferentiation has been assessed previously, the mechanisms underlying biochemical and molecular status of tMSCs with impacted pathways and proteins remain relatively unknown.

Transdifferentiated MSCs show morphological and molecular changes similar to native SCs. Studies have characterized this process by immunocytochemical, Western blot, enzyme-linked immunosorbent assay (ELISA), reverse transcription-polymerase chain reaction (RT-PCR), and functional bioassays [15,24,28]. However, in prior studies, no biochemical and molecular profiles of the tMSCs were uncovered. To this end, we used proteomic and system-based pathway analyses to identify highly regulated proteins present during transdifferentiation. Such investigations uncovered pathway regulation for tMSCs into SC-like cells and the morphological and biochemical changes reflective of the biological outcomes. S100 expression, nerve growth factor and brain derived neurotrophic factor (NGF and BDNF) secretion and promotion of neurite outgrowth were demonstrated. Proteomic analyses revealed significant changes in protein regulation (387 out of total 808) and identified pathways associated with axonal guidance, myelination, neural development and neural growth and differentiation. Further investigation will begin to unravel the functional state of tMSCs for neuroregeneration.

2. Materials and methods

2.1. Isolation of bone marrow-derived MSCs

Mesenchymal stem cells were isolated and cultured from Brown Norway rats (*Rattus norvegicus*) [24,37]. Isolated cells were propagated in maintenance media containing alpha minimum essential media (αMEM, Gibco BRL Thermofisher Scientific, Waltham, MA, USA) supplemented with 20% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA, USA), 2% 200 mM solution GlutaMAX (Gibco BRL), and 1% antibiotic–antimycotic (Invitrogen Thermofisher Scientific, Waltham, MA, USA) and maintained in incubators under 5% CO_2 atmosphere at 37 °C. The sub-culturing of the cells was performed every 2 to 3 days as described in our previous work [24].

2.2. Transdifferentiation of MSCs (tMSCs) into Schwann-like cells

The protocol for *in vitro*, tMSCs into SC-like cells, was modified from a published report by Dezawa and colleagues [15]. The protocol was previously used to produce SC-like cells on micropatterned substrates [24]. Briefly, MSCs were propagated under normal growth conditions to a confluency of 30–40% before being subjected to 1 mM β-mercaptoethanol (BME; Sigma–Aldrich, St. Louis, MO, USA) in αMEM for one day. The following day, media from the flask was aspirated completely and cells were washed with phosphate buffered saline (PBS) before subjecting them to αMEM media supplemented with 10% FBS and 35 ng/ml all-trans-retinoic acid (ATRA (R2625); Sigma). After three days of incubation in retinoic acid-supplemented medium, cells were washed again with PBS and further subjected to αMEM media supplemented with 20% FBS, 14 μl forskolin (FSK; EMD Millipore, Billerica, MA, USA), 5 ng/ml platelet-derived growth factor (PDGF; Sigma), 10 ng/ml basic fibroblast growth factor (bFGF, Promega Corporation, Madison, WI, USA) and 200 ng/ml heregulin β1 (HRG; Calbiochem, EMD Millipore) for 8 days *in vitro* (DIV).

2.3. Immunocytochemistry (ICC) and ELISA tests

Transdifferentiation was assessed by examining protein expression after ICC using an image-based high throughput imaging system. tMSCs and undifferentiated MSCs (uMSCs) were plated in 96 well plates (655090, Greiner Bio-One, Monroe, NC, USA) at a density of 2000 cells per well at day 12 of transdifferentiation. Cells were washed with 0.1 M PO_4 buffer twice and then fixed for 20 min in 4% paraformaldehyde in 0.1 M PO_4 buffer. After fixation, cells were washed with PBS three times every 10 min and incubated in blocking solution prepared using PBS containing 5% normal donkey serum (NDS, Jackson ImmunoResearch, West Grove, PA, USA), 0.4% bovine serum albumin (BSA; Sigma) and 0.2% Triton X-100 (Fisher Scientific) for 1 h. Antibodies specific to glial, neuronal and proliferation markers (Table 1) were diluted to desired concentrations in blocking solution and applied to the cells overnight at 4 °C. The following day, cells incubated with primary antibodies were washed with PBS thrice every 10 min before applying secondary antibodies Donkey-α-Mouse-Cy3 (1:500, Jackson ImmunoResearch) or Donkey-α-Rabbit-Cy3 (1:500, Jackson ImmunoResearch) along with DAPI (1:50) for 60–90 min in the dark. Finally, as the last step, cells were washed with PBS again thrice every 10 min to remove any non-bound secondary antibody. 200 μl of PBS was left in each well for fluorescence imaging. Plates were imaged using an ImageXpress Micro high content screening system (Molecular Devices, Sunnyvale, CA, USA) and percent immunostaining for each antibody was quantified using the multi wavelength cell scoring module of the MetaXpress software (Molecular Devices, Sunnyvale, CA, USA).

A nerve growth factor-β (NGFβ) ELISA Kit (ab100757, Abcam, Cambridge, MA, USA) and a BDNF ELISA kit (BDNF Emax® ImmunoAssay System, Promega, Madison, WI, USA) were used to quantify these secreted neurotrophic factors released from tMSCs and uMSCs. ELISAs were performed based on the instructions provided by the kit manufacturer. Cells were plated in 6 well plates at an initial plating density of 30,000 cells/well. After two days, conditioned media samples were collected, and cells were fixed and stained with DRAQ5 (nuclei stain) (ab108410, Abcam) to assess the number of cells present at the time of sample collection. A Student *t*-test was performed to determine significant differences in the data obtained for immunocytochemistry and ELISA tests.

2.4. PC12-TrkB propagation and cell function

Genetically modified BDNF receptor expressing PC12-TrkB cells (a gift from M. Chao, New York University) were grown in RPMI-1640 Medium (30-2001, ATCC, Manassas, VA, USA), supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum, under 5%

Please cite this article as: A.D. Sharma, et al., Proteomic analysis of mesenchymal to Schwann cell transdifferentiation, J Prot (2017), http://dx.doi.org/10.1016/j.jprot.2017.06.011